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The Journal of LABORATORY and CLINICAL MEDICINE

The Official Publication of the Central Society for Clinical Research

Volume 58 | Number 5

November, 1961

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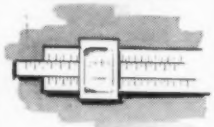

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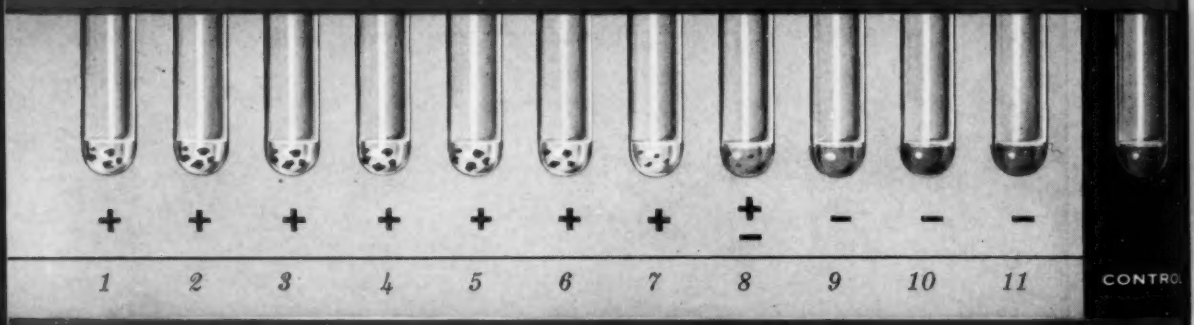

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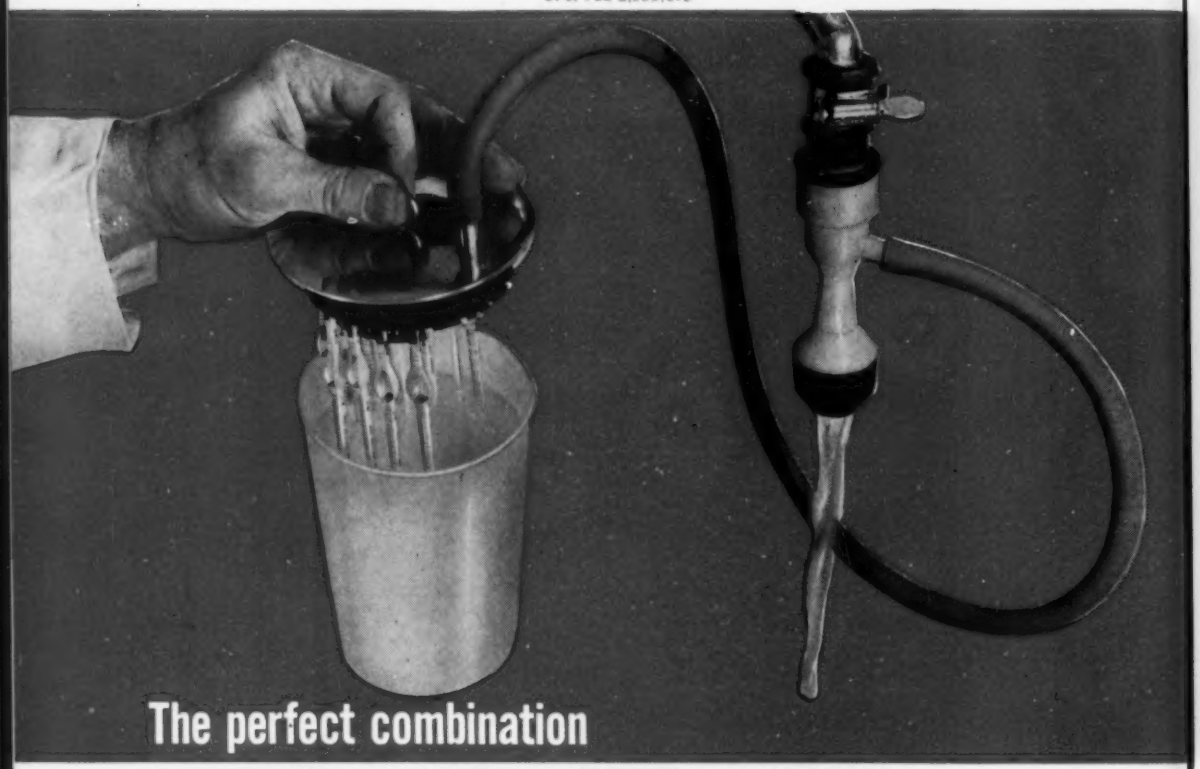

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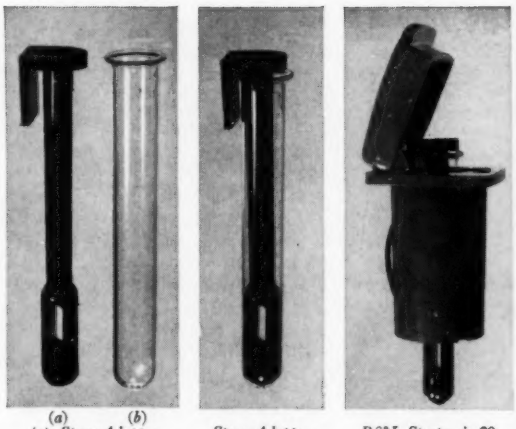

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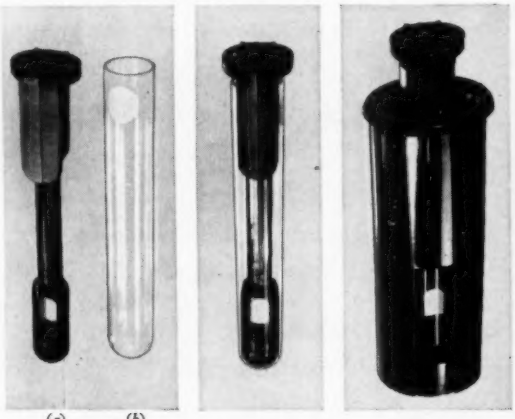

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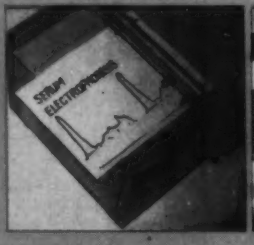

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[1]Lathe, G. H. (Sass-Kortsak, Andrew, ed.) in **Kernicterus;** report based on symposium held at IX International Congress of Paediatrics, Montreal, July, 1959. University of Toronto Press, Toronto, 1961.

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This astronomical figure, according to a recent note** in the Journal of the American Medical Association, is—theoretically—the number of individual combinations of blood factors which could arise from the 60 different blood factors which have been identified in man. Only a few laboratories, however, have access to more than 20 of the known reagents, which limits the theoretical number of currently identifiable combinations to about a million. Another limiting factor is the tendency of blood factors within the various "systems" to be inherited in blocks. But the possibilities are still enough to stagger the imagination.

Figures such as these dramatize the highly responsible role played by present-day medical technology. Science has come a long way since the 1930's, when virtually the only pretransfusion testing was the identification of the four ABO main blood groups.

Today's medical technologists are dealing with a "changed population." The millions of blood transfusions administered in recent years have caused sensitizations that make the task of grouping, typing and crossmatching bloods constantly more complex and demanding. And, too, the identification of sensitizations due to pregnancy often calls for the highest measure of technical skill.

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**Wiener, A. S., Blood Types, J.A.M.A. *177*: 737 (Sept. 9) 1961.

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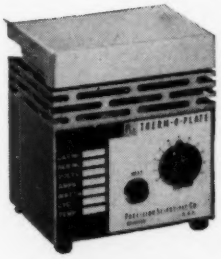

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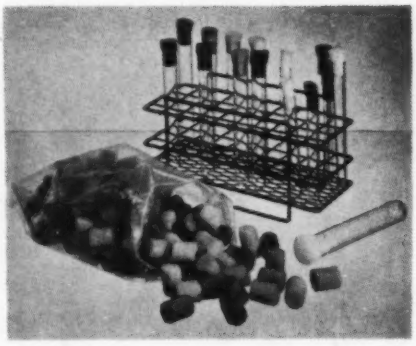

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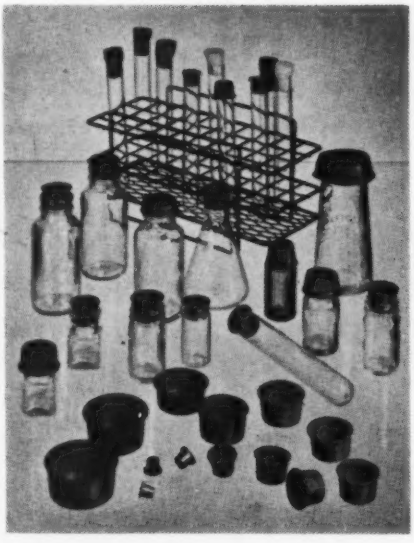

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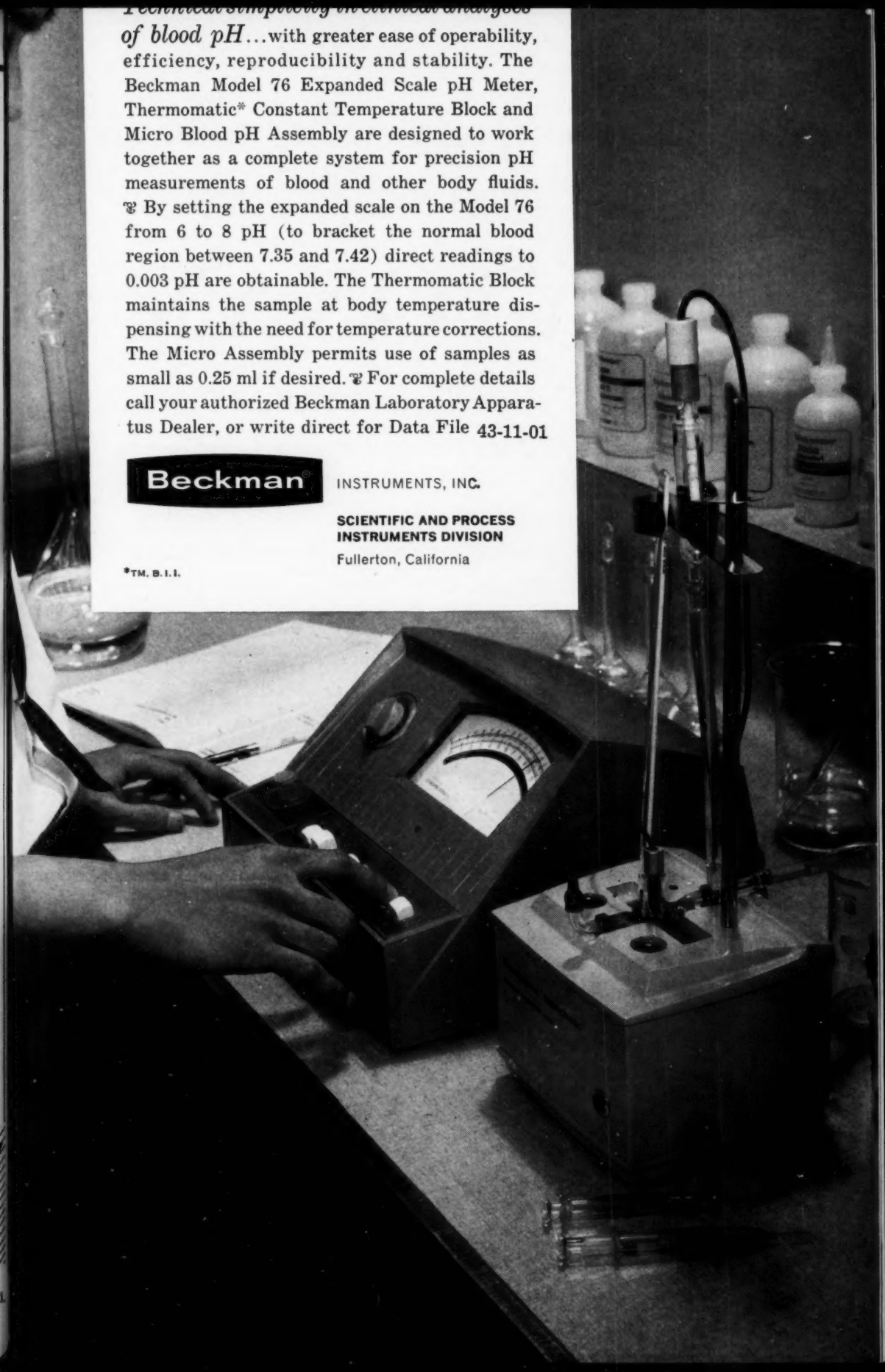

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CYTOTOXIC AGENT for palliative chemotherapy of certain types of malignant neoplasms

Cytoxan demonstrated therapeutic advantage over other agents in a recent 12-month study* of 130 patients, most of whom were refractory to previous treatment:

DISEASE	NUMBER OF PATIENTS	RESULTS				INADEQUATE TRIAL
		GOOD	FAIR	TRANSIENT	FAILURE	
Lymphoma	74	34	3	5	23	9
Hodgkin's Disease	29	10	3	4	9	3
Lymphosarcoma	21	15	0	0	3	3
Multiple Myeloma	16	9	0	0	4	3
Reticulum Cell Disease	8	0	0	1	7	0
Leukemia	23	10	0	0	8	5
Chronic Lymphatic Leukemia	8	4	0	0	3	1
Acute Monoblastic Leukemia	11	5	0	0	3	3
Acute Myeloblastic Leukemia	4	1	0	0	2	1
Carcinoma (Breast, Lung, and Solid Tumors)	29	2	1	1	23	2
Miscellaneous (Mycosis, Fungoides, Psoriasis)	4	0	0	1	3	0
Total	130	46	4	7	57	16

Adapted from Wall, R. L., and Conrad, F. G.

Note that the neoplastic disorders most responsive to Cytoxan were lymphosarcoma, multiple myeloma, Hodgkin's disease, and chronic lymphatic leukemia. Occasionally, good results were observed in acute monocytic leukemia and carcinoma of the breast.

Other advantages noted in this study*

• multiple routes of administration, permitting prolonged maintenance therapy • lack of latency period for bone marrow depression • failure to produce significant thrombocytopenia • potential therapeutic effect in diseases usually unresponsive to other mustard compounds (*e.g.*, myeloma).

*Wall, R. L., and Conrad, F. G.: Arch. Int. Med. *108*:456-482, 1961.

INDICATIONS: Cytoxan is valuable for palliative therapy of certain malignant neoplasms, particularly some of those arising in the reticuloendothelial and hematopoietic systems and certain solid tumors.

Types of cancer which have proved relatively more susceptible or more resistant to Cytoxan therapy may be grouped as follows:

Group I: Neoplasms relatively susceptible to Cytoxan
- Hodgkin's disease
- Lymphomas: lymphosarcoma; giant follicular lymphoma; reticulum cell sarcoma
- Leukemia: acute; chronic
- Mycosis fungoides

Group II: Neoplasms relatively resistant to Cytoxan
- Malignant neoplasms of the breast and the ovary*
- Malignant neoplasms of the lung, the gastrointestinal tract and the genitourinary system, including the cervix and the uterus
- Malignant neoplasms of miscellaneous origin
- Malignant melanomas

*Malignant tumors of these organs are somewhat more susceptible to Cytoxan therapy than are the others included in this group.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan*—Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2 to 3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan—Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage—The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2 to 3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

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Cytoxan Tablets for oral administration, 50 mg., white, round tablets, flecked with blue for easy identification. Packaged, 100 tablets per bottle.

For a copy of the Cytoxan brochure, or other additional information on Cytoxan, communicate directly with the Medical Department, Mead Johnson Laboratories, Evansville 21, Indiana.

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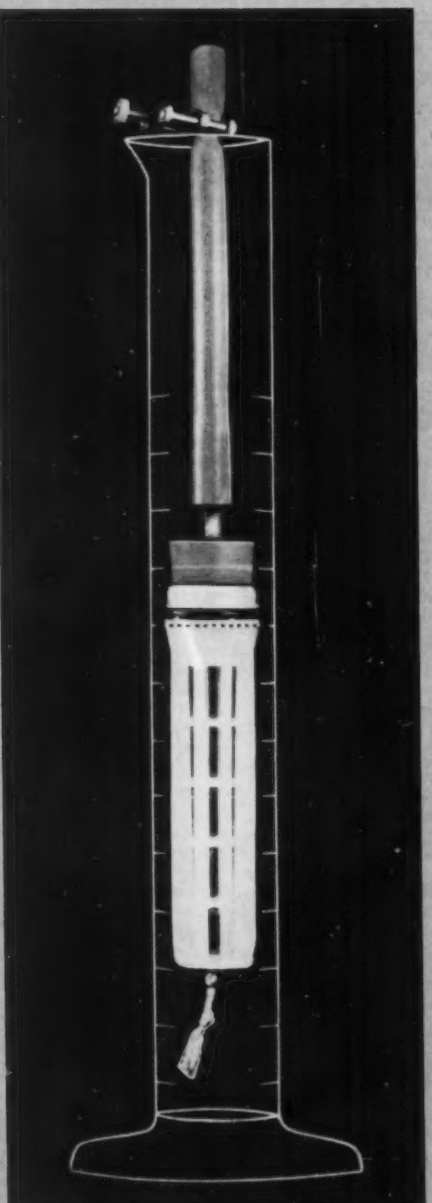

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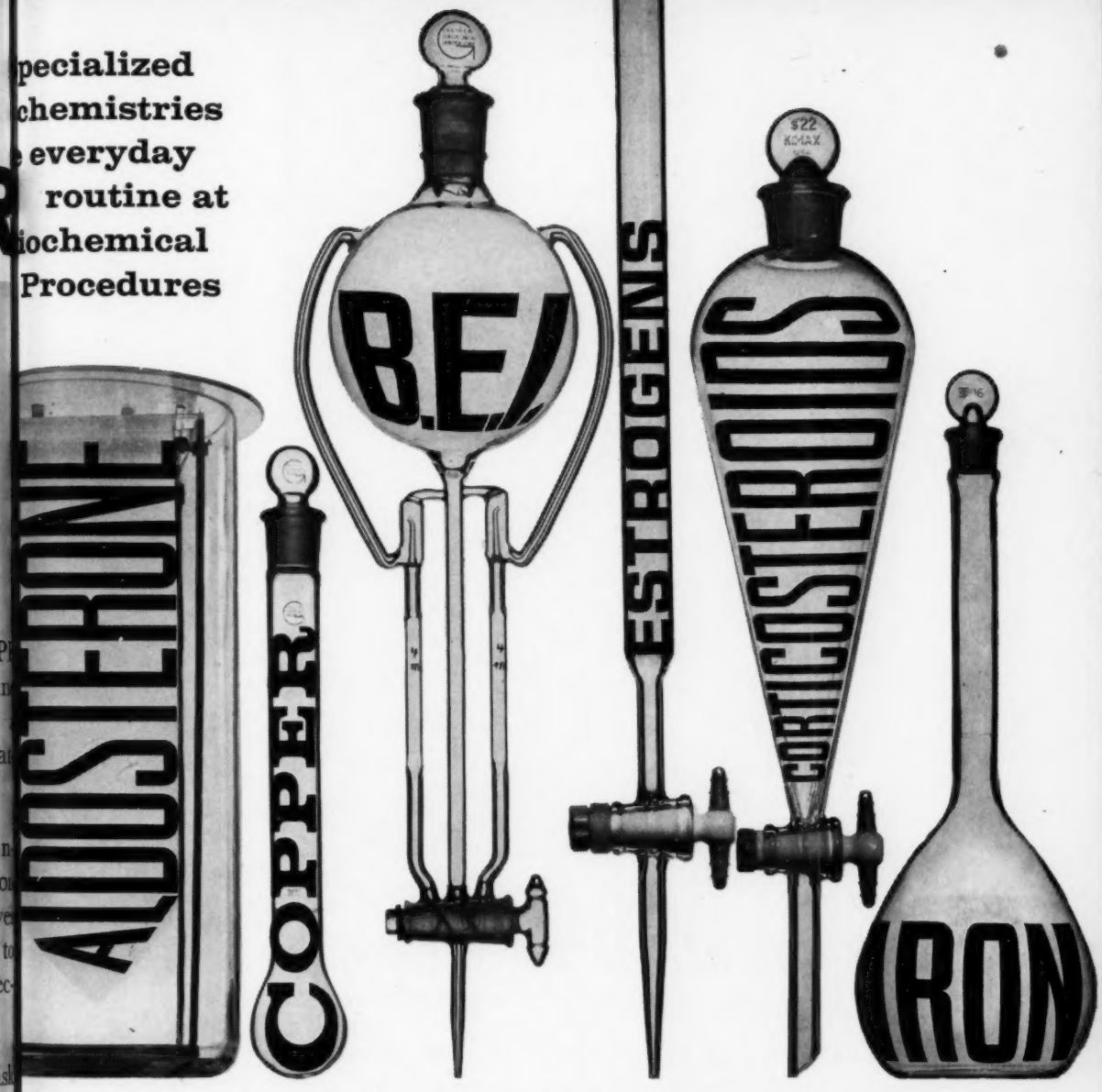

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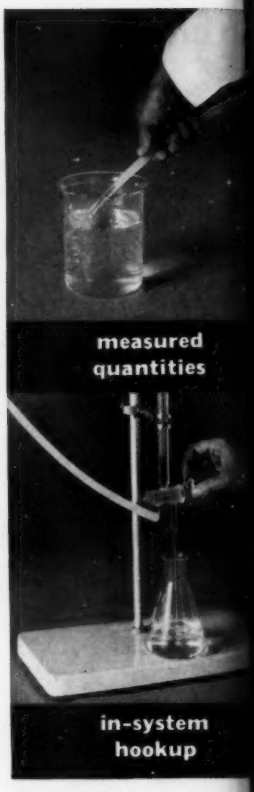

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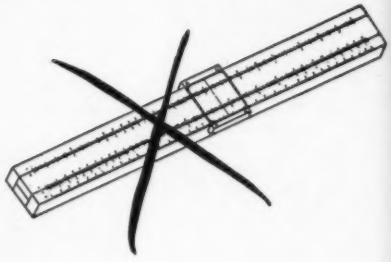

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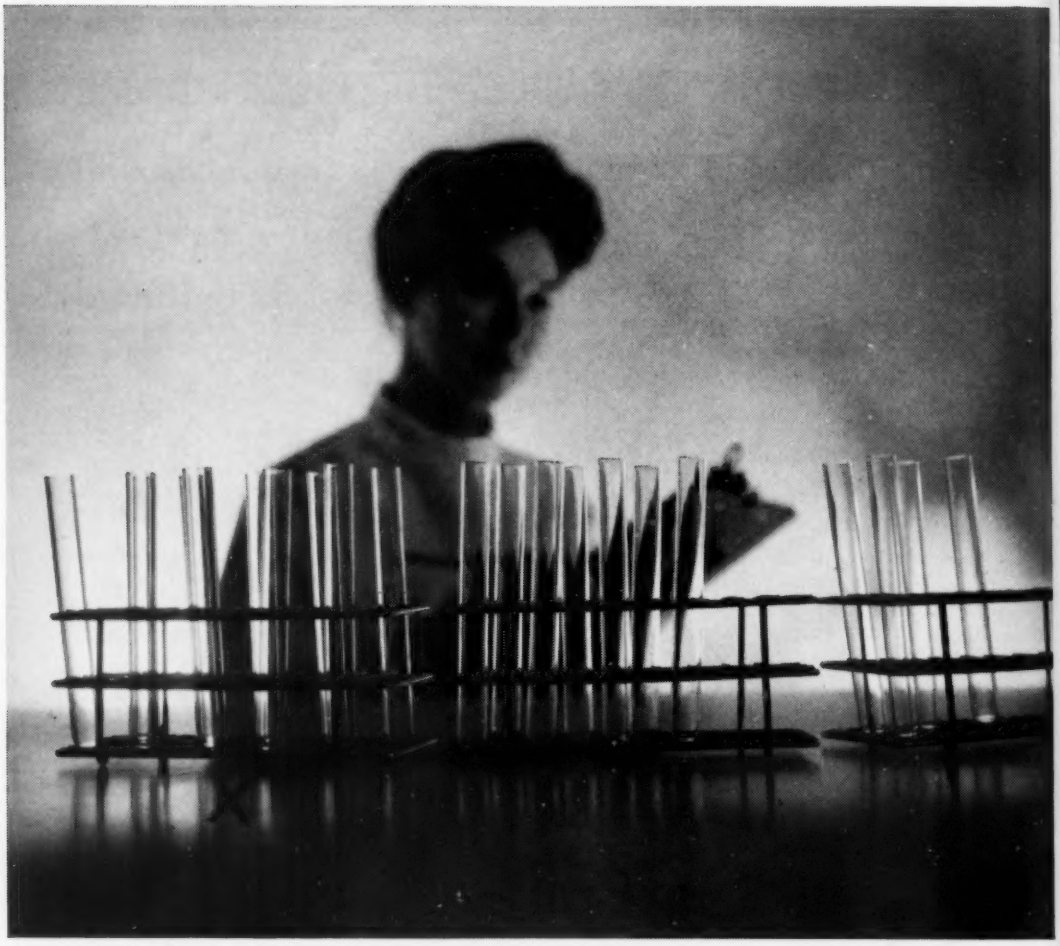

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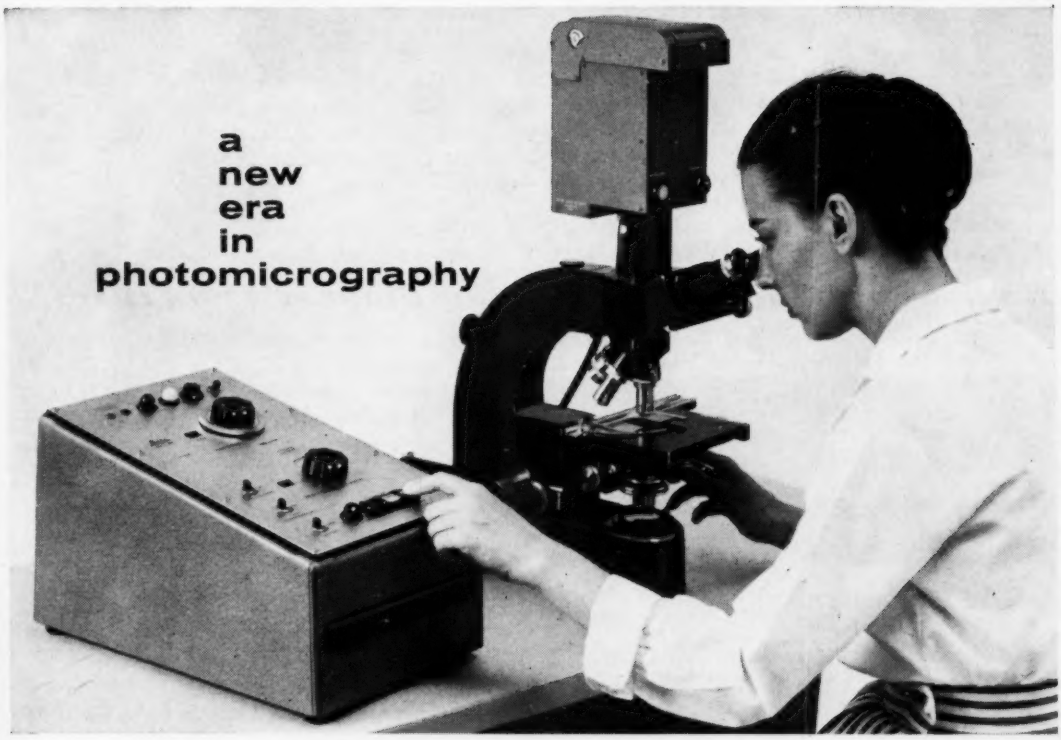

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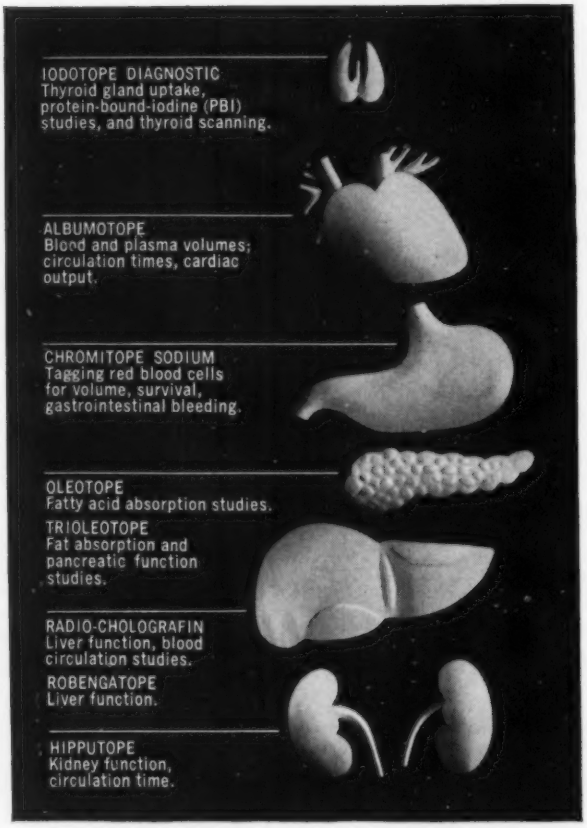

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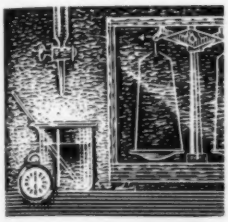
a glance at yesterday in relation to today



IN NOVEMBER – (1850) – *American Journal of Science and Arts* reports a talk by A. Voelcker in which he attempts to show that there is considerable inaccuracy in the usual estimation of the nutritive qualities of food (based upon determination of total nitrogen). The food under discussion is a fungus called *Agaricus pruness*, which is highly edible and remarkable for forming most beautiful fairy rings. Protein was separated by precipitation with basic acetate of lead, and nitrogen determined by combustion. Results indicate that less than 5 per cent of the nitrogen in the dry fungus exists as proteine compounds and nearly one-third is ammonia or some other form without nutritive value.[1]

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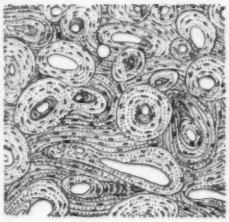

IN NOVEMBER – (1937) – Thannhauser and his co-workers[2] report some studies on the effects of thiol compounds on phosphatase activity of human serum. In a previous paper they had reported a marked activation of serum phosphatase by ascorbic acid. Now, cysteine and reduced glutathione are shown to produce a sharp decrease in phosphatase activity and to neutralize ascorbic acid activation. This deactivation occurs in serum taken from normal individuals and from patients with Paget's disease (a bone disorder characterized by high serum phosphatase levels). However, these impressive deactivating effects of cysteine cannot be reproduced *in vivo*.

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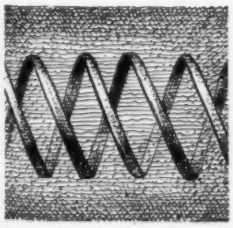

IN NOVEMBER – (1956) – the Watson-Crick structure of DNA is slightly modified. They had assumed that one of four pairs of complementary purines and pyrimidines (adenine-thymine, thymine-adenine, guanine-cytosine, and cytosine-guanine) is present at each level of two intertwining polynucleotide chains. Each pair was presumably linked by two hydrogen bonds. The model provides a postulate for the duplication of genes: the two polynucleotide chains could separate, and each might serve as a template for replication. Now, study of crystal structure data by Pauling and Corey indicates that adenine and guanine should be linked by *three* hydrogen bonds. This corresponds to a higher degree of specificity and therefore strengthens the Watson-Crick theory of gene duplication.[3]

If you are studying gene duplication or nucleic acid structure you will find a wide selection of nucleic acid precursors on the new Schwarz BioResearch list, including many labeled with C^{14} or H^3. Send for our new catalog and price list.

1. Voelcker, A.: On the percentage of nitrogen as an index to the nutritive value of food. Am. J. Sc. *10*:403 (Nov.) 1850.
2. Thannhauser, S. J.; Reichel, M.; Grattan, J. F., and Maddock, S. J.: Studies on serum phosphatase activity. IV. The deactivating effect of thiol compounds and bile salts on serum phosphatase activity in vitro and in vivo. J. Biol. Chem. *121*:721 (Nov.) 1937.
3. Pauling, L. and Corey, R. B.: Specific hydrogen-bond formation between pyrimidines and purines in deoxyribonucleic acids. Arch. Biochem. *65*:164 (Nov.) 1956.

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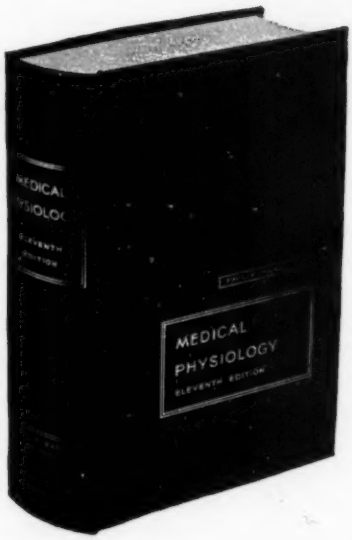

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Edited by PHILIP BARD, Professor of Physiology, The Johns Hopkins University. With 15 collaborators. Published August, 1961, 11th edition, 1339 pages, 7¼" x 10½", 508 illustrations, 4 in color. Price, $16.50.

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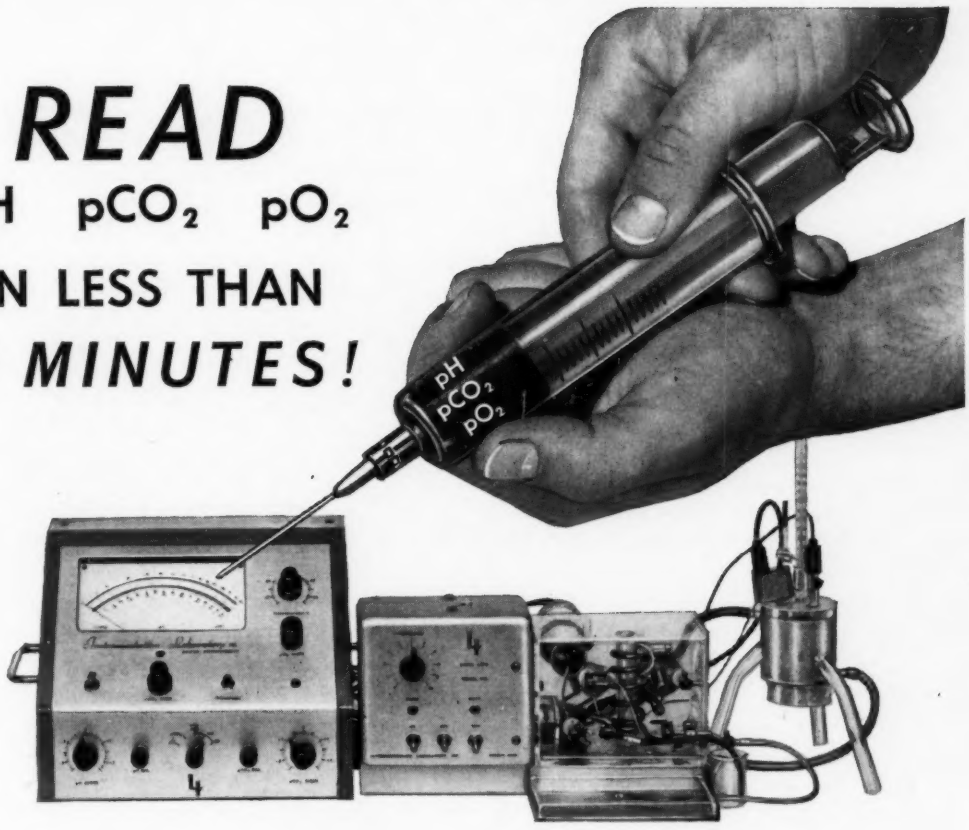

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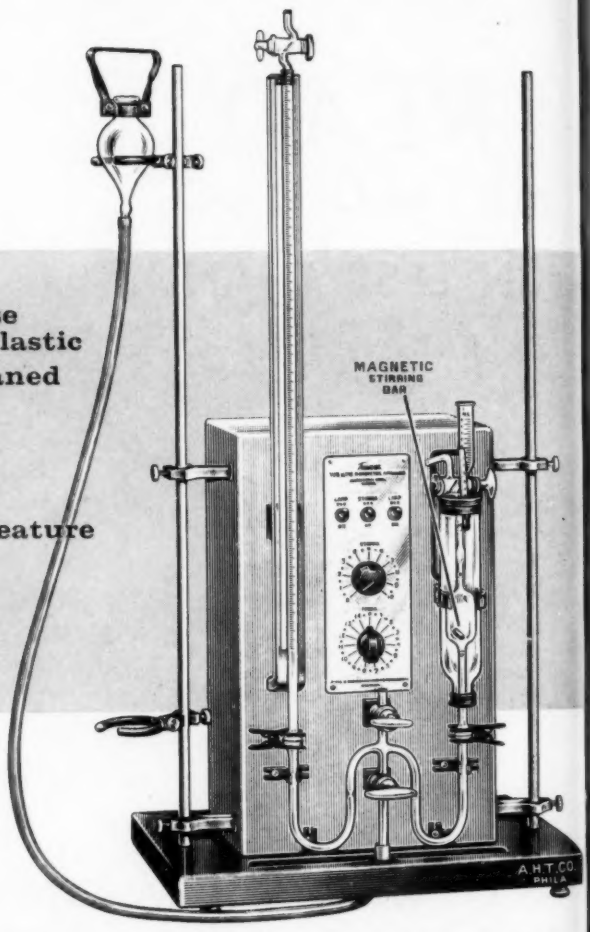

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Volume 58 Number 5 NOVEMBER 1961

Clinical and experimental

Studies on blood lipids during exercise. II. The arterial plasma-free fatty acid concentration during and after exercise and its regulation

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It has been established during recent years that peripheral tissues, including muscles, can oxidize fatty acids. It also has been shown that the plasma-free fatty acids are a rapidly mobilized and transported form of fat. The relationship between carbohydrates and lipids in generating energy for muscular work is not known. These facts initiated the present studies on the free fatty acid fraction during exercise, the results of which are reported herein. Our previous finding that free fatty acids decrease in plasma during short periods of exercise has been confirmed and extended. During prolonged exercise experiments the decrease of the free fatty acids was significant up to and including 10 minutes of exercise; thereafter, no significant changes from the resting level were observed. After exercise an extremely rapid increase was observed, the free fatty acids being doubled within 5 minutes. It was shown that the decreased free fatty acid concentration was related to an increased flux of free fatty acids from plasma. Furthermore, a linear relationship existed between the decrease of free fatty acids and the increase in pulse rate during exercise. The suggestion is made that the increase of blood flow during exercise caused the increased outflow of free fatty acids from plasma.

Our studies on the behavior of the blood lipids during exercise were stimulated by the fact that at the present complete knowledge is lacking concerning the contribution of lipids to the energy metabolism in muscular tissues. Changes

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Supported by a grant from the Swedish Medical Research Council.

Based on a report read before the Swedish Society of Clinical Physiology, Stockholm, December, 1959.

Received for publication Sept. 7, 1960.

from the resting state occurring during exercise might throw some light on these problems. The work of Andres and associates[1] had suggested that carbohydrates play a minor role in the metabolism of muscle at rest. In agreement with this, evidence was produced as early as 1913 by Lafon,[2] that lipids were extracted by muscular tissues. Further evidence in support of this view was obtained by Bing and his group[3] and later by Gordon.[4] Gordon showed that, in man, the beating heart extracted free fatty acids (FFA). This FFA fraction has in recent years been shown to be a readily available and rapidly metabolized lipid fraction.[5] Thus it might be expected that this fraction of the blood lipids is of importance for the energy metabolism.

It was shown in the first paper of this series[6] that the arterial concentration of FFA* decreased during short-time exercise and also that the exercising leg extracted FFA. This decreased systemic FFA concentration might be due to a decreased rate of addition of FFA to plasma, to an increased rate of elimination from plasma, or both. This question has been studied in the present paper and evidence has been obtained that the decrease in FFA concentration is intimately correlated to the increase in blood flow during exercise, and furthermore, that it is mainly due to an accelerated outflow of FFA from plasma. After the completion of this work, Friedberg and colleagues[7] confirmed our earlier finding that the FFA concentration decreases during exercise. Furthermore, this group presented data showing that the elimination rate of plasma-FFA was increased during exercise.

Materials and methods

Subjects. Twenty-two men between 20 and 60 years of age were studied. Sixteen were in good health without evidence of disease as judged from their history and clinical and laboratory examinations. This group included young as well as older subjects. Data for 4 of these 16 men were taken from an earlier publication.[6] The remaining 6 were patients with peripheral arteriosclerosis and symptoms of intermittent claudication; they had no evidence of any other metabolic disease and were able to work.

Long-time exercise test at constant work load. This test was performed by 6 healthy subjects in the morning after they had fasted over-night. The exercise was performed in the sitting position on a bicycle ergometer. The work was started at 600 Kg. per minute and continued for 30 minutes. In some cases it was then increased to 1,200 Kg. per minute, and the subjects continued the exercise as long as they were able. Repeated blood samples, usually taken every fifth minute, were drawn into heparinized syringes from a catheter inserted into the brachial artery.

Short-time exercise test at increasing work loads. The test was done by the 10 normal men as well as by the 6 patients in the morning after they had fasted over-night. The exercise was performed on a bicycle ergometer and consisted of an uninterrupted series of work, increasing the work load 150 to 300 Kg. every six minutes. The work usually was performed in the sitting position and sometimes in the supine position. The exercise generally was continued until the subject had performed his utmost. In some cases the exercise was repeated after a resting period of 30 minutes. Blood was withdrawn into heparinized syringes from a catheter inserted into a brachial artery, and the samples were taken during the last minute of the 6 minute period.

FFA determination. The blood was centrifuged and duplicate plasma samples were pipetted into methanol within 5 minutes after withdrawal. The lipids were extracted with

*FFA is synonymous with the earlier used term, UFA.

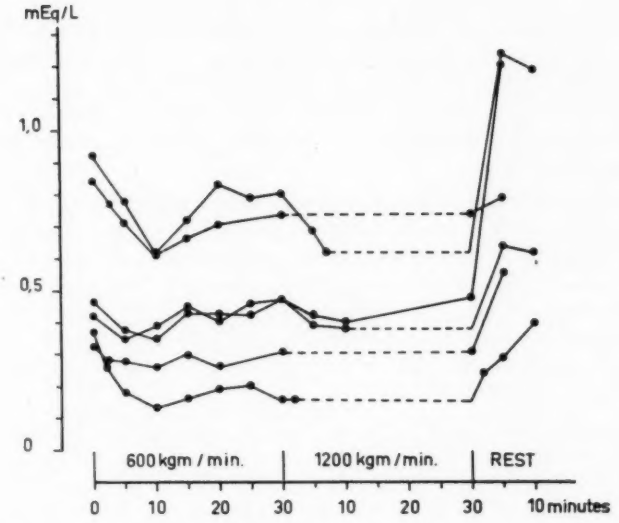

Fig. 1. Arterial plasma concentration of FFA in 6 normal men during exercise at a constant work load of 600 Kg. per minute. Four of the men performed exercise at 1,200 Kg. per minute after the first exercise. Values corrected for changes in protein concentration.

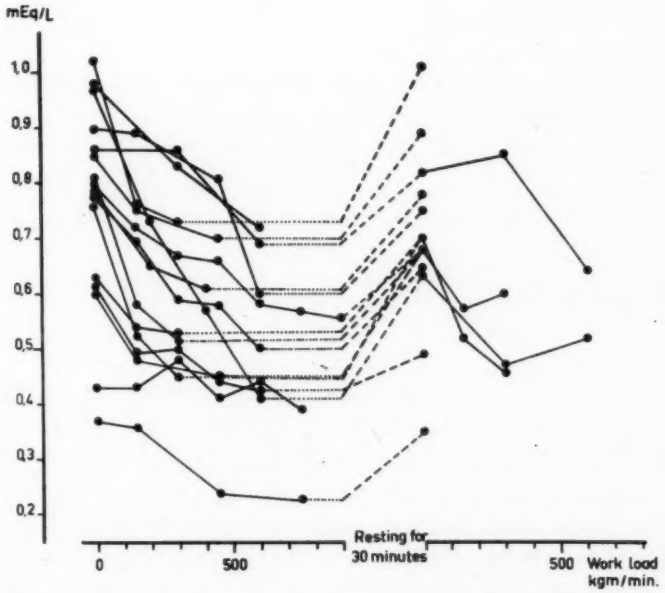

Fig. 2. Arterial plasma concentration of FFA in 10 normal men and 6 patients with arteriosclerosis obliterans during exercise for 6 minutes at increasing work loads. The blood samples were taken at the fifth minute of each period of exercise. Values have not been corrected for changes in protein concentration.

chloroform-methanol; the extract was washed with acid phosphate, and the phospholipids were separated on silicic acid, as described earlier.[8] Triplicate 5 ml. aliquots of the chloroform phase obtained after the silicic acid chromatography were pipetted off. One milliliter of a Nile blue A indicator solution (90 per cent methanol, 10 per cent water) was added and the fatty acids were titrated with 0.02 N sodium hydroxide in methanol, delivered from an Agla microburette. With this technique such short-chain acids as pyruvic and lactic do not interfere, nor does added heparin.

Plasma protein concentration. Total plasma protein was determined in duplicate according to the biuret method. No definite changes occurred in arterial plasma protein concentration during exercise performed in the supine position. In patients who exercised in the sitting position, however, the protein concentration generally increased about 10 per cent. The FFA concentration has been corrected for the changes in plasma protein concentration when the correlation between FFA concentration and pulse rate was studied. The plasma volume changes occurring during exercise performed in the sitting position do not change the validity of the results obtained.

Isotope experiment. This was done in one healthy subject. Palmitic acid-1-C^{14},* dissolved in the recipient's own plasma, was given as a constant infusion before, during, and after exercise. Two days before the experiment the healthy recipient was given 100 Gm. of glucose in the morning; after about 1 hour blood was withdrawn and citrate plasma was prepared and stored in the cold. The palmitic acid, containing about 11 μc per milligram, was dissolved in an equivalent amount of 0.02 N sodium hydroxide by warming and was added to the plasma (about 1 mg. to 100 ml. of plasma) ½ hour before the experiment. Eighty milliliters of this plasma was given as a constant intravenous infusion at a rate of 1 ml. per minute. This corresponds to an inflow of about 0.1 mg. FFA per minute. The rapid turnover of plasma-FFA means that about one third leaves the plasma each minute. In order to obtain a constant specific activity, 3 ml. of the active plasma was injected immediately before the start of infusion. Blood samples were drawn every fifth minute during a resting period of 30 minutes and at different intervals during and after exercise. The blood lipids were extracted and treated as described above. The FFA fraction was isolated from the neutral lipid fraction by partition between light petroleum ether and 50 per cent alkaline ethanol in 4 funnels as described by Borgström.[9] After isolation of the FFA 15 mg. of palmitic acid was added to minimize sublimation during evaporation. The fatty acids were dissolved in toluene containing 300 mg. per cent diphenyloxazole and the activity was estimated in a liquid scintillator.† At least 10,000 counts were counted. The possibility of contamination of the isolated FFA fraction with lower glycerides was studied in one fraction. After passage through IRA 400 and recovery of the acid fraction[8] no significant change in activity was observed.

This experiment was performed with the patient in the supine position. No changes were observed during exercise in the arterial plasma protein concentration.

Results

Prolonged exercise at constant work load. In all 6 cases the FFA concentration decreased during the first period of exercise (Fig. 1). However, after 10 minutes of exercise at 600 Kg. per minute, the FFA showed a tendency to increase again. As can be seen from Table I, the decrease was statistically significant at 2, 5, and 10 minutes after commencement of the exercise. Thereafter the FFA level was not significantly different from the pre-exercise level. In the 3 patients who continued the exercise at 1,200 Kg. per minute for 5 minutes or more the FFA level dropped once again.

*The Radiochemical Centre, Amersham, England.
†Ecco Electronics, Southend-on-Sea, Essex, England.

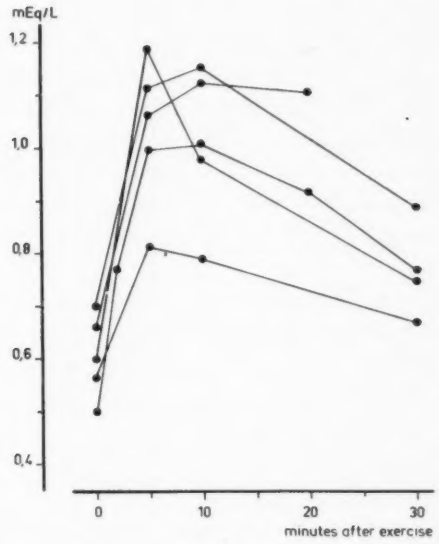

Fig. 3. Arterial plasma concentration of FFA after exercise in 5 men. Values are corrected for changes in protein concentration.

Short exercise at increasing work loads. The FFA level dropped during each increment of exercise (Fig. 2), the decrease being significant already at the first load (0.10 ± 0.03 mEq. per liter, $p < 0.01$). In the 4 patients who repeated the exercise after a resting period of 30 minutes a decrease was observed once again. No significant difference was observed between the normal men and the patients with peripheral arteriosclerosis; thus the data have been treated together.

FFA concentration after exercise. Figs. 1 and 2 show that the FFA concentration increased after the exercise. The time relationship for this increase has been studied in more detail in 5 cases. Fig. 3 reveals that the increase after exercise was extremely rapid, the FFA level being about doubled in 5 minutes.

Isotope experiment. The constant infusion of the labeled palmitic acid resulted during rest in a rather constant amount of C^{14} in the isolated FFA fraction. During exercise the total amount of C^{14} of this fraction rapidly decreased in order to increase rapidly after exercise. In Fig. 4 the plasma FFA concentration

Table I. Time relationship of individual changes in plasma-FFA concentration during exercise at a work load of 600 Kg. per minute

Time of exercise (minutes)	2	5	10	15	20	25	30
Mean value (mEq./L.)	−0.07	−0.09	−0.14	−0.08	−0.07	−0.04	−0.03
Standard error of the mean (mEq./L.)	0.01	0.02	0.05	0.05	0.03	0.05	0.04
No. of individuals	3	6	6	6	6	4	6
Statistical significance (p)	< 0.05	< 0.01	< 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Changes have been calculated from the resting value and are expressed in milliequivalents per liter. Negative sign indicates a decrease from the resting FFA level.

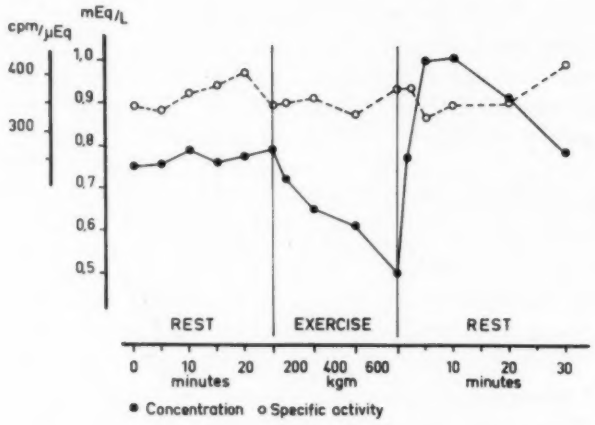

Fig. 4. Arterial plasma concentration of FFA and specific activity in the isolated FFA fraction before, during, and after exercise during constant infusion of albumin-bound palmitic acid-1-C^{14}. The exercise was performed for 6 minutes at each work load; the first sample at 200 Kg. per minute was taken after 2 minutes of work and all the others were taken after 5 minutes at each load.

as well as the *specific activity* of this fraction is given. As is seen, the FFA concentration decreased about 40 per cent during exercise and increased about 100 per cent in 5 minutes after exercise. In spite of these pronounced changes in the FFA concentration, the *specific activity* of the isolated FFA fraction did not show any significant changes, indicating that the outflow from plasma was the factor regulating the FFA concentration during the exercise test, while the rate of addition to plasma of FFA probably remained rather constant during the experiment.

Discussion

FFA concentration during exercise. The plasma-FFA concentration decreased significantly during the first 10 minutes of exercise at a constant work load. Furthermore, when exercise was performed for a short time at successively increased work loads, the FFA level decreased at each new work load.* This decrease of the FFA may be ascribed to either a reduced rate of addition to plasma or an increased removal rate from plasma. Most of the plasma-FFA are derived from the adipose tissue glycerides and the mechanism most often discussed in connection with the regulation of the FFA level is the flow from the adipose tissue to plasma.[5] Thus it has been shown that the decrease in plasma-FFA after insulin administration is related to a decreased output from the depots in vivo[10] and from adipose tissue in vitro.[11] Results indicating similar mechanisms for the FFA lowering effect of glucose also have been obtained.[11, 12] On the other hand, it has been shown that epinephrine and starvation, both increasing plasma-FFA, increase the FFA output from adipose tissues.[11, 13] The

*In most subjects several pre-exercise samples were drawn. They never differed more than 0.05 mEq. per liter (see also Fig. 4 concerning this pre-exercise constancy).

possibility that the FFA decrease during exercise might be regulated via the fat depots was discussed earlier.[6] One point favoring this hypothesis was that evidence had been obtained suggesting that the blood flow through skin and adipose tissue was reduced during exercise in order to allow maximal blood flow through the working muscles.[14] However, data obtained on arteriovenous differences of FFA during exercise[6] suggested that the lowered FFA level was due to an extraction of FFA by the exercising leg, although the interpretation of such data is difficult due to the fact that the arteriovenous difference is measured across muscular as well as adipose tissues.

However, during exercise the most typical change from the resting state is the increase in blood flow. The possibility that the FFA decrease was related to the increase in blood flow was tested by plotting the decrease of FFA during short periods of exercise at increasing work loads against the increase in pulse rate (Fig. 5). These data were obtained from 5 normal individuals and were corrected for changes in total plasma protein concentration during exercise. It is evident from Fig. 5 that the decrease of plasma-FFA was linearly related to the increase in pulse rate ($r = 0.80$, $p < 0.001$). The equation for the regression of the changes of FFA concentration (Y, in milliequivalents per liter) on the changes of pulse rate (X, in beats per minute) was found to be $Y = -0.0025\ X - 0.06$, with a sample standard deviation from regression of 0.05 mEq. As the cardiac output seems to vary directly with the pulse rate,[15] this indicates that the FFA concentration during exercise was directly related to the blood flow. The possibility has recently been discussed by Havel and Goldfien[16] that the rate of blood flow might influence the removal of FFA. Our data indicate a direct relationship between the blood flow and the removal rate of plasma-FFA. Such a mechanism appears to fit available data and hypotheses about the transport of the FFA from the vascular to the extravascular pools by means of the binding of the FFA to serum albumin and to receptor sites on cell surfaces.

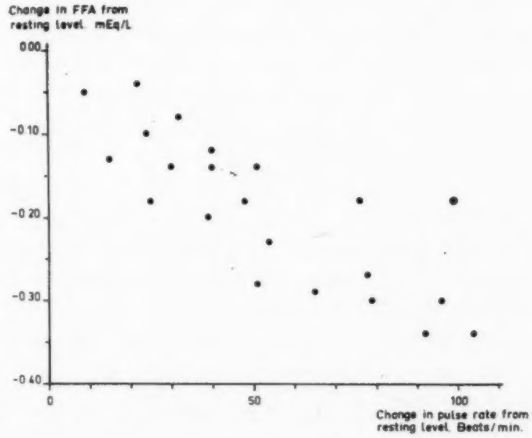

Fig. 5. Relationship between changes of FFA concentration and of pulse rate during exercise. Values from 5 normal men performing exercise for periods of 6 minutes at increasing work loads.

Any increase in blood flow, regardless of which tissues are perfused, would increase the amount of FFA-albumin complex passing the capillary walls per unit time and might thus facilitate the transcapillary transfer of the fatty acids.

It can also be concluded from the experiment with constant infusion of albumin-bound palmitic acid-1-C^{14} that the flux of this acid from plasma was increased during exercise. The constant specific activity of the FFA fraction, furthermore, indicates that the flow of FFA from the depots remained relatively constant during the period of exercise. This interpretation requires the assumption that palmitic acid is representative of the entire FFA fraction. This fraction is composed mainly of palmitic, oleic, and stearic acid,[17] and it was found by Fredrickson and Gordon[18] that the 2 acids have similar turnover rates. In this connection the recent finding of Friedberg and associates[7] is of greatest interest. These authors found that the plasma-FFA fraction decreased during acute vigorous exercise, which is in agreement with our results. They also determined the half-life time of the equilibration phase of the FFA fraction in the subjects at rest and during the early phase of exercise. It was shown that the mean half-life time decreased from 2.36 at rest to 1.46 minutes during exercise. The possible mechanism of this increase in disappearance rate was no discussed. The increased disappearance of FFA is consonant with our findings from constant infusion of palmitic acid before and during exercise. Furthermore, the technique with constant infusion showed that the output from the depots appeared to be unchanged during the early phases of exercise. On the other hand, this output might well be changed after more prolonged exercise, as the FFA level tended to increase and approach the resting level when exercise was performed for longer than 10 minutes (Fig. 1 and Table I).

FFA concentration after exercise. The increase in the FFA level after exercise also was found in our earlier study[6] and Friedberg and associates[7] made the same observation. This increase is so rapid that it will in all probability invalidate studies not performed with indwelling catheters and with samples taken *during* the exercise. The results of experiments using constant infusion of palmitic acid seem to imply that this increase was due to a decreased removal of the FFA from plasma when the exercise ended. It might well be that this increase in FFA is related to the decrease of blood flow after exercise.

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Effect of epinephrine on lactate, pyruvate, and excess lactate production in normal human subjects

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The present study is concerned with the effects of epinephrine on 2 indices of the adequacy of tissue levels of molecular oxygen, namely the ratio of lactate and pyruvate, and excess lactate production. The results indicate that in normal man a portion of the hyperlactacidemia associated with epinephrine administration may be ascribed to an increase in anaerobic carbohydrate metabolism and a portion to an increase in aerobic metabolism.

The relationship between lactate and pyruvate (equation *1*) depends upon lactate dehydrogenase (LDH) activity and the ratio between reduced

$$\text{Pyruvate} + \text{DPNH}_2 \underset{}{\overset{\text{LDH}}{\rightleftarrows}} \text{lactate} + \text{DPN} \qquad (1)$$

($DPNH_2$) and oxidized (DPN) diphosphopyridine nucleotide. Since molecular oxygen is required to maintain the normal ratio between $DPNH_2$ and DPN, the equation is driven to the right during anaerobiosis in the intact organism, with a consequent accumulation of blood lactate above and beyond that which can be ascribed to any concurrent increase in pyruvate. Elevated levels of lactate also can occur under aerobic conditions in which there is an increase in carbohydrate metabolism. Under such aerobic conditions, the increase in lactate will be accompanied by a concurrent increase in pyruvate. In order to differentiate the hyperlactacidemia due to anaerobic from that due to aerobic causes, use of the lactate: pyruvate ratio has been employed.[1, 2] A more precise method, as suggested by Huckabee,[3] consists of calculating the "excess lactate" (XL) production, or in other words, calculation of the amount of lactate formed which cannot be due to increased pyruvate metabolism alone but which must also, in

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Supported by Research Grant No. H-3359 from the National Heart Institute of the National Institutes of Health, United States Public Health Service.

Received for publication Jan. 16, 1961.

part or in whole, be due to impaired activity of the LDH system by either altered LDH activity or the absence of amounts of oxygen adequate to maintain the normal $DPNH_2$: DPN ratio. Excess lactate is calculated according to

$$XL = (L_n - L_o) - (P_n - P_o)\ (L_o/P_o) \qquad (2)$$

equation (*2*), where L_o and P_o are, respectively, control values for lactate and pyruvate, and L_n and P_n are their experimental values.

In the course of studying the effects of general anesthetics on blood lactate, pyruvate, and excess lactate production in humans, it was noted that certain anesthetics, notably diethyl ether and cyclopropane, were associated with elevations of blood lactate and pyruvate and with significant excess lactate production.[4] These anesthetics are also known to be associated with increased sympathoadrenal activity.[5] The relationship between catechol-amine release and metabolic changes during anesthesia will be discussed in a separate paper. The present study was instituted after a review of the literature indicated that, although epinephrine administration has long been known to result in hyperlactacidemia and elevated blood levels of pyruvate,[6] data are not available which permit calculation of excess lactate production during epinephrine administration. Those few reports in which lactate and pyruvate have been studied simultaneously following administration of epinephrine[7-12] are inadequate for determination of this point, usually because peripheral venous blood was used or because, the object of the experiments being other than study of excess lactate formation, the data are presented in such a manner as to preclude use of equation (*2*) above. Peripheral venous blood samples in such studies reflect primarily changes in metabolism of the peripheral organs (usually skin and muscle) from whence the venous blood is draining. The over-all metabolic response of the entire organism can be evaluated only by use of mixed venous or arterial blood samples.

Materials and methods

Five normal, nonfasting adults, 4 men and one woman, weighing 52 to 84 kilograms and aged 28 to 38 years, were studied in the supine position while breathing room air. Weight was determined on a metabolic scale accurate to 10 Gm. A fresh solution of epinephrine in normal saline (4.0 mg. per liter) was prepared for each subject. The possibility could not be excluded that some norepinephrine was present as a contaminant in the epinephrine used. The norepinephrine which could have been present, however, was so small that, after dilution, at the rates of administration used, the maximum amount of norepinephrine which could have been administered would have been 0.007 μg per kilogram per minute. This amount of norepinephrine, if present, is below that amount found in this laboratory necessary to produce detectable cardiovascular responses in humans. It is also below the amount which must be infused intravenously in order to produce metabolic changes.[6] The results, therefore, cannot be ascribed to norepinephrine effect.

In each experiment, an intravenous infusion of normal saline (0.037 ml. per kilogram of body weight per minute) was administered for 30 minutes into an arm vein with the subject in the supine position in order to achieve a resting state. After 30 minutes, an arterial (brachial) blood sample was obtained by direct puncture of the ipsilateral arm after infiltration of a local anesthetic (1 per cent lidocaine) about the artery. Following this, epinephrine infusion was started through the same intravenous needle at a rate such that 0.15 μg of epinephrine was administered per kilogram of body weight per minute, the rate of

Table I. Effect of epinephrine on lactate, pyruvate, and excess lactate production (mM. per liter of whole arterial blood)

Subject No.	*Control*		*Experimental*		*"Excess lactate"**
	Lactate	*Pyruvate*	*Lactate*	*Pyruvate*	
1	0.767	0.172	1.644	0.202	0.740
2	0.344	0.128	1.333	0.165	0.891
3	0.844	0.126	2.278	0.169	1.145
4	0.467	0.104	1.867	0.165	1.130
5	1.278	0.084	2.911	0.147	0.667

*Derived from equation *(2)*.

saline administration remaining constant. The epinephrine infusion was continued for 20 minutes, at which time the second arterial blood specimen was obtained by direct puncture of the contralateral brachial artery. After the second specimen was obtained, the epinephrine infusion was discontinued. The amount of epinephrine infused has been shown to have significant cardiovascular effects.[13]

Each arterial blood sample was placed within 20 seconds in chilled 10 per cent trichloroacetic acid and was then centrifuged, the supernatant being removed and analyzed in duplicate for lactic acid by the method of Barker[14] and for pyruvic acid by the method of Friedmann and Haugen.[15]

Results

Results are summarized in Table I. It can be seen that in all cases epinephrine administration produced elevations of control blood lactate levels. Pyruvate levels also were increased. Because each subject showed an increase in both lactate and pyruvate, and because in each instance the rise was greater than the 5 per cent error inherent in the determinations, the changes are significant. However, the increase in lactate was greater than that which could be explained on the basis of an increased level of pyruvate. This excess lactate, therefore, indicates alteration in the normal activity of the LDH system. The excess lactate cannot be ascribed to decreased utilization of lactate by liver or peripheral tissues. Because equation *(1)* is reversible, an increase in blood lactate due to a decrease in tissue lactate utilization would be associated with a concurrent increase in blood pyruvate, much as an increase in blood lactate due to intravenous infusion of lactate is associated with increased blood pyruvate levels.[16, 17]

Discussion

Epinephrine, when administered to normal humans at the rate of 0.15 μg per kilogram per minute, resulted in a rise in blood levels of both lactic and pyruvic acid. The rise in pyruvic acid is a reflection of the over-all increase in aerobic carbohydrate metabolism associated with increased activation of phosphorylase produced by epinephrine.[6] This rise in pyruvate accounts in part for the concurrent elevation of blood lactate. However, the rise in lactate is greater than can be attributed purely to the rise in the pyruvate, the difference being measured as excess lactate production. Epinephrine itself, although being reported as having a direct effect on lactate dehydrogenase,[18] probably has no

direct effect on this enzyme.[19] The excess lactate production during epinephrine infusion can best be ascribed to alteration of the normal ratio between $DPNH_2$ and DPN, i.e., during epinephrine administration amounts of molecular oxygen are inadequate at the cellular level to maintain normal aerobic carbohydrate metabolism.

Tissue oxygen tensions are a function of the rate at which oxygen is delivered to tissues, the rate at which oxygen is consumed by tissues, and the coefficient of oxygen diffusion in tissues. Little is known regarding alterations of the latter, but epinephrine has a well-recognized ability to increase oxygen consumption.[6] This in itself could lead to a decrease in tissue oxygen tension, assuming an unaltered rate of delivery of oxygen to tissue. But epinephrine also has cardiovascular effects consisting of increased cardiac output and peripheral vasodilation as well as vasoconstriction. The vasoconstriction associated with epinephrine could, even in the absence of concurrent increases in oxygen consumption, result in decreased tissue oxygen tensions. Even the increased rate of delivery of oxygen to tissues suggested by an epinephrine-induced increased cardiac output and peripheral vasodilation could be accompanied by decreases in tissue oxygen tensions if the increase in tissue oxygen consumption produced by the epinephrine were proportionately greater than the increase in tissue blood flow. Although the present results indicate that the over-all response of normal human beings to epinephrine is accompanied by decreases in tissue oxygenation sufficient to impair normal carbohydrate metabolism so that excess lactate is formed, it is impossible to determine whether this is the result primarily of decreases in the rate of delivery of oxygen to tissues, the result primarily of increased rate of oxygen consumption, or whether both factors are contributory. Similarly, the present results, being based as they are on arterial blood samples, do not indicate the specific organ or organs in which tissue oxygen tensions are decreased during epinephrine administration. It has been demonstrated that cutaneous oxygen tensions decrease during epinephrine infusions,[13] but it is likely that other organs more important biochemically will be identified in which tissue oxygen tensions decrease during epinephrine administration. This is presently being investigated by combining polarographic techniques and arteriovenous lactate and pyruvate levels across individual organs.

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Nitrous oxide intracardiac shunt detection during inhalation anesthesia

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The nitrous oxide test is an improved method for detection and localization of left to right circulatory shunts. To add this diagnostic test to the armamentarium of the cardiopulmonary laboratory presented problems in the catheterization of children and infants, as these procedures were performed during inhalation anesthesia. It was desirable to retain (1) reversibility and moment to moment controlability of the depressant agents, (2) rapid awakening and return of protective reflexes, as well as (3) a steady reproducible state, as provided by inhalation techniques.

Without sacrificing the advantages of inhalation anesthesia, the choice of agents was altered to halothane and air, and the anesthetic machine was utilized to deliver the nitrous oxide test mixture.

The nitrous oxide test data for 10 anesthetized patients (26 determinations) were compared with similar data for 12 unanesthetized patients (25 determinations). There was no evidence of interference with the diagnostic nitrous oxide test by the concomitant halothane-air anesthesia. At the same time the cardiac catheterizations were facilitated by this anesthetic technique.

The nitrous oxide test is an improved method for detection and localization of left to right circulatory shunts.[1, 2] The basis for this test is the rapid movement of nitrous oxide from the arterial blood into the body tissues until a saturated or equilibrated state exists. During the short period immediately following the institution of nitrous oxide inhalation and before tissue saturation occurs, there is an appreciable difference in arteriovenous concentrations of the gas. The delayed appearance of nitrous oxide in the right-heart blood is due to rapid tissue absorption. If a left to right shunt is present, nitrous oxide–enriched blood from the left heart raises the level in the right-heart blood, making the

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Received for publication Jan. 19, 1960.

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detection of shunts possible. By obtaining blood samples from the right atrium, right ventricle, or pulmonary artery by means of a catheter, such a shunt may be not only detected but localized.

The value of this test has been demonstrated amply by Sanders and Morrow,[1, 2] who reported their results in a total of 88 patients with intracardiac shunts and 176 control subjects, who were patients with rheumatic heart disease without shunt. The superiority of this method over the classical oxygen method also has been reported by these authors, who showed that, in 149 patients in whom both tests were performed, there were 22 diagnostic errors on the basis of oxygen differences and only 3 errors with the nitrous oxide test.[1] In addition, the ratio of pulmonary to systemic flow determined by the nitrous oxide method has correlated well with metered flow measurements in dogs with experimentally induced intracardiac shunts. Therefore, the technique is valid for determining the magnitude of a left to right shunt.[3]

For several years in this institution, inhalation anesthesia has facilitated cardiac catheterization in children without interference with the conventional oxygen method of shunt detection. The anesthetic management, described elsewhere,[4] utilized nitrous oxide as the primary anesthetic agent, thus precluding the simultaneous use of nitrous oxide as a test gas. Since the improved method of shunt detection was desired, another anesthetic technique was sought which would not interfere with the nitrous oxide diagnostic test. At the same time it was desirable to maintain the advantages of inhalation anesthesia, namely: (1) moment to moment controlability and reversibility of the anesthetic state, thus giving increased safety to the patient, (2) a steady and reproducible state, and (3) rapid awakening and resumption of nutritional intake.[4]

In eliminating nitrous oxide as the primary anesthetic agent, other nonflammable inhalation agents were considered. Trichlorethylene-air anesthesia had been reported to be less than ideal because of the frequency of tachypnea, tachycardia, and cardiac arrhythmias.[5] Chloroform was discarded because of its profound myocardial effects[6] and, historically, hepatic toxicity.[7] With the recent introduction of another halogenated, volatile anesthetic, halothane,[8] it was believed this drug might present a means of satisfying the desired requirements. Although other investigators independently have utilized essentially the same anesthetic management[9-11] the authors are not aware of the nitrous oxide diagnostic test being performed during inhalation anesthesia elsewhere. Morrow and colleagues[1] reported performance of this test during "general anesthesia"; however, these patients were not anesthetized with inhalation agents.[12]

Materials and methods

The anesthetic management described below has been utilized for all pediatric cardiac catheterizations whether or not the nitrous oxide test was employed. Twenty-six nitrous oxide determinations in 10 patients are included in this report.

Fluids. As the catheterizations were performed in the early afternoon, the children were allowed to have a clear liquid breakfast (Jello, strained fruit juice, etc., but *no* milk), then nothing by mouth for the 4 or 5 hours preceding anesthesia. The small infants were allowed their feedings up until 3 hours prior to anesthesia, the last feeding consisting of 5 per cent glucose in water in place of the usual formula.

Premedication. Each patient received scopolamine, 0.08 to 0.12 mg., 90 minutes prior to catheterization. Meperidine (1 mg. per pound up to 50 mg.) was added in children over 2½ years of age. The narcotic dosage was decreased a total of 5 to 10 mg. in the more severely ill patients.

Preparation. A stethoscope was taped to the precordium and, to older children, a blood pressure cuff was applied. Electrocardiograph electrodes were attached to the older children, most of whom were already familiar with this apparatus. The precordial stethoscope was removed at the time of fluoroscopy.

Induction. Anesthesia induction was begun by administering halothane-air plus oxygen (total, 40 per cent oxygen) with a bag and mask. The halothane* was delivered from a vaporizer† in gradually increasing concentrations up to 2.5 per cent. Total flow rate was at least 6 L. per minute. As soon as consciousness was lost, the electrocardiograph electrodes, if not already in place, were attached, and the tracing was monitored throughout the procedure on a cathode ray oscilloscope.

Intubation. When the depth of anesthesia was adequate for laryngoscopy, the larynx and vocal cords were sprayed with 6 per cent cocaine. Exposure of the vocal cords was usually done with ease as halothane diminishes laryngeal reflexes even in light anesthesia.[13] Because of rapid awakening, the anesthetic mixture was readministered by bag and mask until laryngoscopy depth was again obtained.

A properly selected endotracheal tube was inserted and connected to a nonrebreathing valve and reservoir bag. The use of muscle relaxants to facilitate tracheal intubation was not found necessary.

Maintenance. Anesthesia was maintained with halothane, 0.75 per cent, and air (4 to 8 L. per minute flow). An increase of halothane concentration was needed rarely and then for only short periods of time.

No pressure was allowed on the breathing bag during right-heart pressure measurements because of the immediate hemodynamic effects.[14]

Catheterization. The procedure was started after the cut-down sites were infiltrated with 1 per cent procaine and the patient's extremities had been taped to the mattress to prevent the slight movements which may occur during very light anesthesia. Blood removed for sampling was replaced as needed via the catheter.

Nitrous oxide test. The anesthetic machine‡ utilized was provided with orifice type water flow meters. A commercial tank of air was attached by a high pressure tubing and a yoke to 1 of 2 nitrous oxide inlets. A tank containing 50 per cent nitrous oxide, 30 per cent nitrogen, and 20 per cent oxygen was attached to the remaining nitrous oxide inlet. Either of these gas mixtures then passed via the nitrous oxide valve and meter through the vaporizer to the patient. In an orifice type flow meter, because of turbulent flow, the density of the gas being measured determines the calibration. The densities of air, the nitrous oxide mixture, and nitrous oxide are 1.20, 1.54, and 1.84 Gm. per liter (at 1 atmosphere and 20° C.), respectively.[15] Thus a setting of 6 L. per minute on the nitrous oxide meter would deliver 7.4 L. per minute of air or 6.5 L. per minute of the mixture. This variation in flow rate is not critical because the vaporizer utilized was flow-compensated and will deliver the same concentration of vapor within flow rates of 5.0 to 10.0 L. per minute. The flow rate must be sufficient to prevent carbon dioxide accumulation, i.e., approximately twice the minute volume.[16]

As the catheterization proceeded under stabilized halothane-air anesthesia, an arterial blood sample for a blank determination was drawn. The valve controlling the air from the tank was closed, and when the flow through the anesthesia machine ceased, the tank containing the 50 per cent nitrous oxide mixture was opened. The same flow rate and halothane

*Halothane was supplied as Fluothane by Ayerst Laboratories, Inc., New York, N. Y.
†Fluotec Mark 2, manufactured by Cyprane Limited, Keighley, England.
‡Foregger Texas Model, manufactured by the Foregger Company, Inc., Roselyn Heights, N. Y.

concentration were maintained. Because of the dead space within the apparatus, e.g., tubing, there was a delay from the time the nitrous oxide mixture was turned on until it reached the endotracheal tube. This time interval (5 seconds) was determined by the timed passage of carbon dioxide at the same flow rate, utilizing an infra-red analyzer. Therefore, 5 seconds after the nitrous oxide mixture was turned on, the timing of the nitrous oxide test was begun. For clinical application, this time interval may be estimated. The method then followed the technique described by Sanders and Morrow.[2] Ventilation was assisted during the test to simulate the deep breathing performed by unanesthetized patients.

Upon completion of the test, the nitrous oxide mixture was turned off and the air valve re-opened, maintaining the same concentration of halothane vapor. After 10 minutes the procedure could be repeated.

Table I. Comparison of nitrous oxide tests in conscious and anesthetized patients

	Conscious subjects	*Anesthetized subjects*
Number	12	10
Number of determinations	25	26
Average arterial blank	1.44 vol. %	1.78 vol. %
(Range)	(0.97 to 2.58)	(1.31 to 2.64)
Average arterial N_2O content	13.35 vol. %	10.14 vol. %
(Range)	(6.37 to 17.19)	(5.46 to 16.92)
Average age	25.4 years	5.7 years
(Range)	(15 to 45)	(2 to 11)

Results

To evaluate the data obtained during inhalation anesthesia, a comparison was made with the results of 25 nitrous oxide determinations performed on 12 unanesthetized patients in the same laboratory and during the same period of time (Table I).

The statistically significant difference (0.34 vol. per cent, $p < 0.001$) in average arterial blank determinations probably was due to the presence of halothane vapor. Methods for determining halothane content in blood were not available at the time of this study. Nevertheless, the stabilized anesthetic concentration was not altered during the test; therefore, it would not interfere with the interpretation of the results.

The average nitrous oxide content in arterial blood was found to be satisfactory in both groups, i.e., over 4.5 vol. per cent in all cases.[2]

One cannot consider these groups to be altogether comparable because of the age differences (25.4 versus 5.7 years). However, no nitrous oxide tests were performed on unanesthetized children.

Five of the 10 patients studied during anesthesia had arterial blood gas analyses (Table II). One patient (A. T.) displayed a decreased oxygen saturation and an increased carbon dioxide content. Repeat studies were done during passive hyperventilation with 35 per cent oxygen, which elevated the oxygen saturation. Dye curves indicated there was no right to left shunt. Another arterial blood study done 5 days later, while the patient was awake, revealed alveolar hypoventilation. The etiology was undetermined despite careful evaluation. The 5 patients not included in Table II had normal arterial oxygen saturation.

Table II. Arterial blood studies in 5 anesthetized patients

Patient	*pH*	*CO_2 (mM./L.)*	*pCO_2*	*O_2 (vol. %)*	*% Sat.*	*Hematocrit*	*Conditions**
M. A.	7.38	16.6	33	18.44	93.1	45.5	Passive hyperventilation
W. M.	7.40	21.4	38	12.63	95.2	31.0	Spontaneous ventilation
D. L.	7.41	21.2	38	11.48	93.9	31.0	Spontaneous ventilation
C. S.	7.34	22.8	46	15.84	95.5	37.9	Spontaneous ventilation
A. T.	—	26.3	—	12.14	84.0	39.0	Spontaneous ventilation
	7.38	24.3	47	13.80	92.2	39.0	35% oxygen, passive hyperventilation
	7.30	26.9	61	13.74	90.9	40.0	Awake, breathing air, 5 days later

*All patients anesthetized, breathing halothane-air mixture, unless otherwise specified.

Table III. Nitrous oxide test data for patients with equivocal values (15 to 20%)

Patient	*Age (years)*	*Nitrous oxide test*			*Diagnosis*
		Sample site	*Vol. % N_2O**	*Ratio (%)*	
D. L.	5	P.A.†	6.12	61.4	Patent ductus arteriosus (confirmed at operation)
		F.A.	9.97		
		R.V.	1.79	18.0	
		F.A.	9.97		
		R.A.	0.90	8.9	
		F.A.	10.12		
C. S.	11	P.A.	1.39	16.9	No shunt. Possible aortic or mitral valvular abnormality
		B.A.	8.20		
		R.V.	0.97	11.9	
		B.A.	8.16		
		R.A.	0.69	8.5	
		B.A.	8.09		
A. T.	4	P.A.	1.44	9.4	No shunt. Primary pulmonary hypertension
		F.A.	15.36		
		R.V.	1.91	16.1	
		F.A.	11.87		
		R.A.	2.11	17.2	
		F.A.	12.26		

*Less blank determination.

†Sample sites: P.A. = pulmonary artery; R.V. = right ventricle; R.A. = right atrium; F.A. = femoral artery; B.A. = brachial artery.

Of the 26 nitrous oxide determinations included in this series, only 4 ratios (in 3 patients) were in the equivocal range (15 to 20 per cent).[2] The nitrous oxide test data for these patients are listed in Table III. Arterial blood studies for these same patients are included in Table II.

The first patient (D. L.) was shown to have a patent ductus arteriosus, with a pulmonary artery pressure of 74/35 mm. Hg. The equivocal nitrous oxide ratio for the right ventricle (18.0 per cent) was interpreted as indicating a relative pulmonary valvular insufficiency, for there was no other evidence of a shunt at the ventricular level. The presence of a blowing diastolic murmur in the pulmonic area, instead of a classical ductus murmur, was consistent with pulmonary valvular insufficiency.

The second patient (C. S.) displayed an equivocal ratio (16.9 per cent) in the pulmonary artery sample, while samples in the right atrium and ventricle revealed no shunt. Satisfactory dye curves (indocyanine green) revealed no shunt. This patient, having been catheterized 2 years previously, has been a diagnostic problem. No evidence of a shunt was found through evaluation of venous oxygen values. This patient has always been asymptomatic, has had normal electrocardiograms and normal heart size radiographically, but did have a pansystolic regurgitant type murmur with normal aortic and pulmonic valve closure. Pressures on either side of the pulmonary valve were normal and the wedge pressure was elevated (14 mm. Hg). Left-heart catheterization was planned to assess the possibility of a mitral or aortic valve lesion.

The third case (Patient A. T.) was ultimately diagnosed as one of primary pulmonary hypertension. This patient was hypoxic, which may have contributed to her elevated pulmonary artery pressure. The cause of her alveolar hypoventilation was not determined but anesthetic management was ruled out (Table II). The nitrous oxide ratios taken from the right ventricle (16.1 per cent) and right atrium (17.2 per cent) were in the lower equivocal range. Technically satisfactory indocyanine green dye injections in the right atrium, right ventricle, and pulmonary artery revealed neither a left to right nor right to left shunt.

Of the 10 patients studied during inhalation anesthesia, 8 of the diagnoses were revealed from the nitrous oxide test alone. Fortunately, other confirmatory evidence was available, making the diagnoses more exact and the prognoses more accurate.

Discussion

Halothane is a potent, nonflammable, nonirritant, volatile anesthetic which has proved to be a useful agent.[13, 17] Considerable care must be taken in its administration because hypotension, myocardial depression, respiratory depression, peripheral vasodilatation, tachypnea, and bradycardia are commonly seen, particularly with overdosage.[13, 17, 18] In patients with heart disease, overdosage may occur at low vapor concentrations, thus careful anesthetic management and accurate vaporization are mandatory. In our hands, halothane-air anesthesia has provided the advantages desired in an inhalation technique.

Keats and associates[5] compared 290 cardiac catheterizations performed on patients during: (1) local anesthesia, (2) trichlorethylene-air anesthesia, and (3) halothane-air anesthesia.[9] They found no difference in mortality or morbidity in the 3 groups. The frequency of deaths during catheterization was related to the severity of the patient's heart disease rather than to the type of anesthetic used. In our own experience with both supplemented nitrous oxide–oxygen and halothane-air anesthesia for cardiac catheterization, there have been no deaths in over 100 procedures. The anesthetic management has not interfered with diagnosis, although in 2 instances low arterial oxygen saturation due to respiratory depression has occurred. Hypoventilation due to anesthesia did not occur in any of the cases in this series.

We gratefully acknowledge the technical assistance of Scott T. Cohen, who performed the blood gas analyses included in this study.

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A study of variables which offer difficulty in the evaluation of the peripheral vascular response to cigarette smoking

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The peripheral vascular responses to cigarette smoking have been studied fairly extensively. However, many studies failed to include a consideration of other factors associated with cigarette smoking which might alter the peripheral blood flow independently of the action of nicotine. Experiments were designed to determine the importance of these factors as possibly contributing to changes in peripheral blood flow.

It was found that one of the most important of these factors was the psychogenic effect of cigarette smoking, since offering a subject a cigarette or lighting the cigarette resulted in peripheral vasoconstriction. This most probably is due to the orienting reflex but may be in part due to a conditioned reflex inasmuch as the anticipation of smoking a cigarette recalls certain learned sensations. Furthermore, the vascular system can be conditioned to constrict to certain stimuli. Sham smoking and deep inspiration also resulted in peripheral vascular constriction. The decrease in digital blood flow with inspiration is highly predictable and cannot be ignored.

Rapid intravenous infusion of nicotine bitartrate resulted in a definite decrease in digital blood flow. Although nicotine produces peripheral vasoconstriction, studies designed to quantitate the decrease in peripheral blood flow due to cigarette smoking must take into consideration other associated factors which may reduce peripheral blood flow.

There has long been interest in the effect of smoking on the peripheral circulation. This interest has been stimulated in part by the possible aggravation, by smoking, of thromboangiitis obliterans and Raynaud's disease. The vasospasm produced by cigarette smoking is considered to be due to the nicotine. Nicotine in low concentrations is known to stimulate the cells of the sympathetic ganglia.[1] It has been shown recently that nicotine can release norepinephrine from chromaffin cells.[2] Although the vasoconstrictive effect

Supported by grants from the United States Public Health Service.

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Received for publication Feb. 8, 1961.

of nicotine itself is well established, experiments designed to study the effect of smoking on the peripheral circulation of intact men often fail to include a study of other factors associated with smoking which may alter peripheral blood flow independently of the action of nicotine. This report summarizes a study of some of the factors other than nicotine which may produce error in experiments designed to measure the effect of cigarette smoking on the rate of peripheral blood flow.

Materials and methods

Fifteen normal men, 6 Caucasians and 9 Negroes, were studied. All were established cigarette smokers who inhaled. The mean age of the subjects was 39 years and the age range was 22 to 51 years. The digital blood flow was measured by the rheoplethysmographic method previously described.[3] The blood flow was recorded for the right index (2RF) and middle (3RF) finger tips at heart level, with the subjects resting comfortably in a hospital-type bed in an air-conditioned room (77° F., relative humidity 49 per cent). The rheoplethysmographic method measures the volumes and rates of digital inflow and outflow continuously throughout a single pulse cycle. Thus, this method obviates the necessity of and eliminates errors concerned with drawing a tangential line through several pulse cycles in an attempt to quantitate blood flow. In addition, it is possible to study quantitatively and continuously the rate of blood flow during both the systolic and diastolic phases of the cardiac cycle. Furthermore, with this method the collecting cuff is placed immediately proximal to the rheoplethysmographic collecting cup in order to measure blood flow more accurately. When the collecting cuff is placed at a distance from the plethysmographic cup, blood trapped between the cuff and the collecting cup accumulates outside of the cup and is not measured, resulting in an indeterminable error.

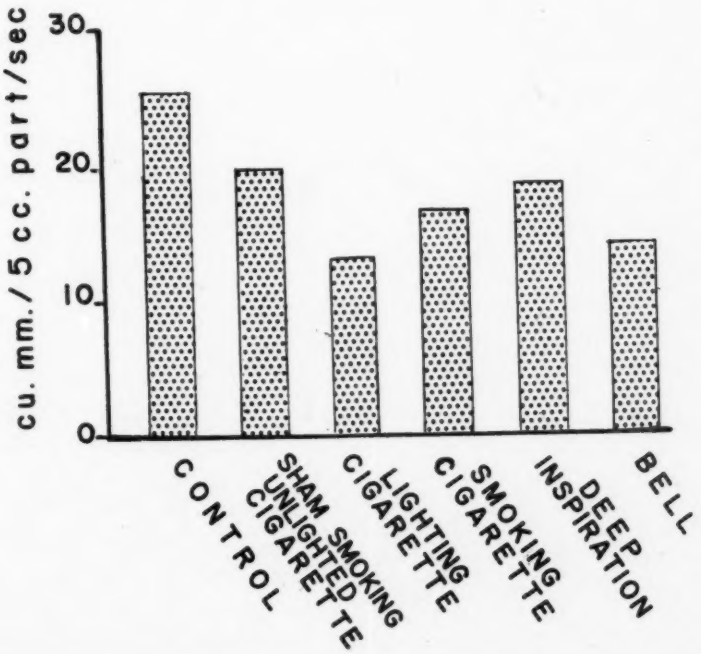

Fig. 1. Influence of various stimuli on the mean rate of blood flow in 10 subjects.

Table I. Summary of data in 10 subjects with statistical analysis*

Patient No.	Control	Offering cigarette		Lighting cigarette		Sham smoking of cigarette		Smoking cigarette		Deep inspiration		Bell	
	Mean rate of flow mm.³ /5 ml. part /second	Mean rate of flow mm.³ /5 ml. part /second	Per cent change	Mean rate of flow mm.³ /5 ml. part /second	Per cent change	Mean rate of flow mm.³ /5 ml. part /second	Per cent change	Mean rate of flow mm.³ /5 ml. part /second	Per cent change	Mean rate of flow mm.³ /5 ml. part /second	Per cent change	Mean rate of flow mm.³ /5 ml. part /second	Per cent change
1	14.5	12.3	15	8.0	45	11.0	24	10.2	30	12.2	16	10.0	31
2	28.4	21.2	25	16.4	42	21.5	24	27.0	5	21.7	24	15.6	45
3	23.6	12.2	48	10.7	54	20.4	14	16.2	31	15.6	34	6.5	72
4	32.2	25.6	20	12.1	62	20.6	36	21.9	31	19.4	39	18.6	42
5	34.2	22.4	34	16.5	51	26.8	22	20.8	39	22.3	34	15.2	55
6	25.4	21.9	14	11.5	54	23.2	9	21.2	17	24.9	2	18.1	29
7	16.1	11.5	29	12.4	23	14.5	10	12.2	24	10.9	32	8.2	49
8	15.1	12.2	19	8.9	41	9.2	39	6.2	59	15.4	+2	13.1	13
9	39.6	22.7	42	18.3	53	32.1	19	16.0	59	27.5	30	18.8	52
10	26.9	18.8	30	15.2	43	21.2	21	13.3	50	19.1	29	20.1	25
Mean	25.6	18.1	28	13.0	47	19.9	22	16.5	34	18.9	24	14.4	41
Standard deviation	±8.1	±5.2		±3.3		±6.6		±6.0		±5.1		±4.5	
Standard error	2.6	1.7		1.1		2.2		2.0		1.7		1.5	
p		$< .05$		$< .01$		$> .10$		$< .02$		$< .10$		$< .01$	

*All values for per cent change are negative except where indicated.

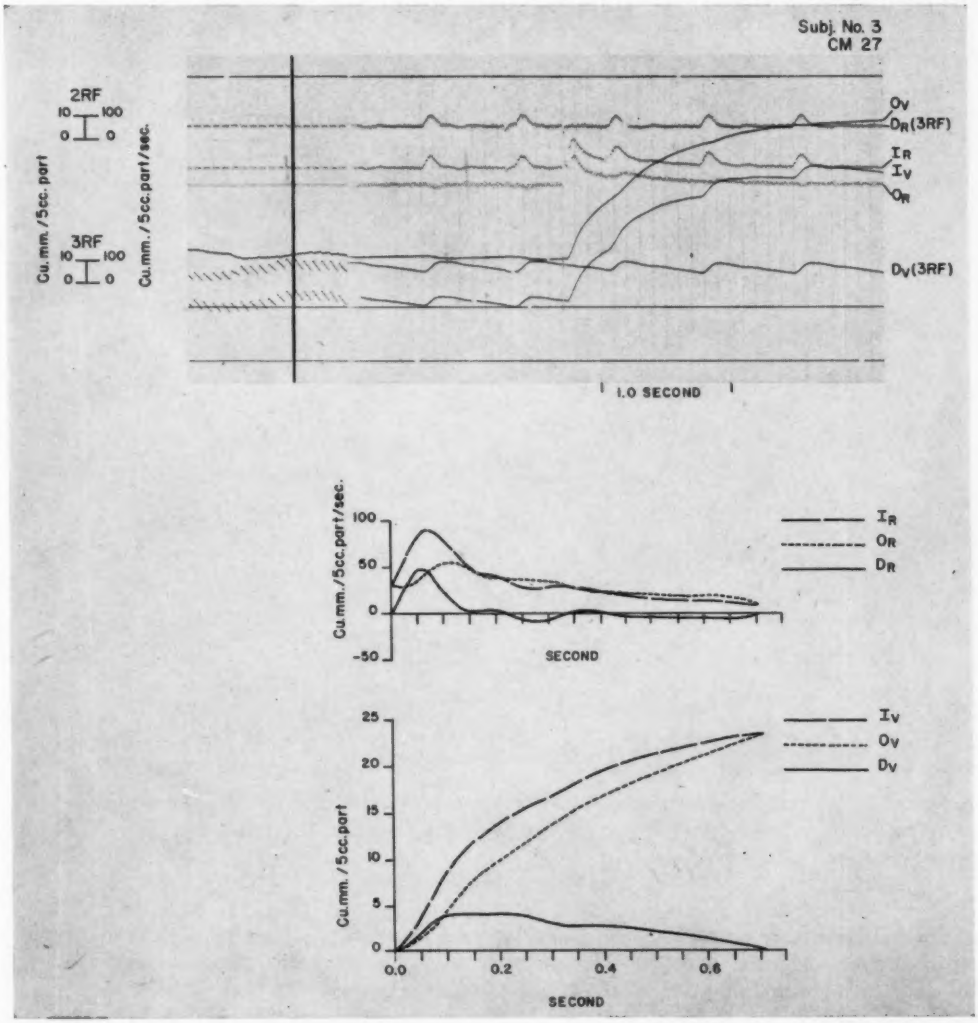

Fig. 2. Digital rheoplethysmogram (RPG) of a normal adult male subject smoking while resting quietly in bed in a comfortable environment. In this, and in all similar illustrations which follow, the original digital RPG recording is shown at the top. The simultaneous time course curves of the volumes and rates of inflow, outflow, and differences between inflow and outflow for a single pulse cycle are shown below. I_V = time course of digital inflow volume; O_V = time course of digital outflow volume; D_V = time course of the difference between inflow and outflow (the volume pulse wave); I_R = time course of the rate of digital inflow; O_R = time course of the rate of digital outflow; D_R = time course of the difference between the rates of inflow and outflow. 2 RF refers to the second right finger and 3RF to the third right finger.

After obtaining control recordings of the volumes and rates of digital blood flow for 10 subjects, measurements were made of the effect on digital flow of the following 4 conditions: (1) offering the subject a cigarette (non-filtered), (2) lighting the cigarette, (3) smoking the cigarette, and (4) sham smoking an unlighted cigarette. The studies were conducted in a quiet room free from outside noises and illuminated with a subdued light. All

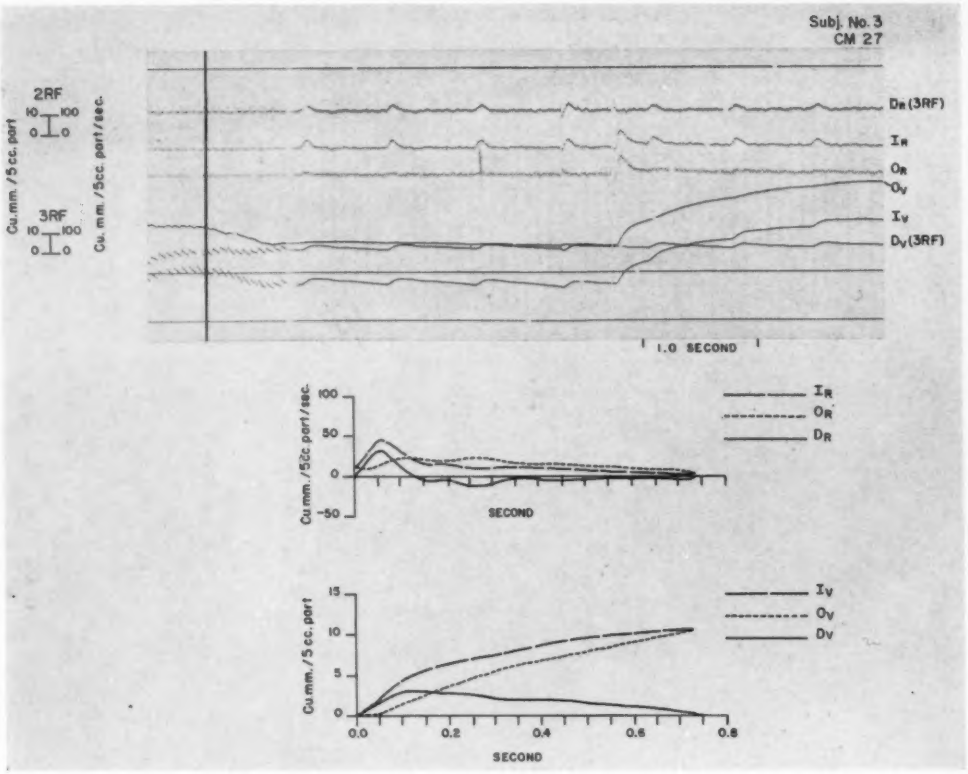

Fig. 3. Digital rheoplethysmogram of the subject in Fig. 2. The RPG was recorded just after inhalation of a lighted cigarette. Consult legend of Fig. 2 for detailed description of these curves and of the symbols.

of the subjects were acquainted with the observers and many had had rheoplethysmograms recorded previously and therefore were familiar with the technique. During sham smoking the subjects were instructed to inhale at a depth and rate similar to that of actual smoking.

In 5 subjects the effect of infusion of nicotine bitartrate on the rate of digital blood flow was measured. During those experiments the arterial blood pressure was recorded directly from the brachial artery by means of a Statham strain gauge and suitable amplifiers and recorders.

Results

1. The influence of offering a cigarette, of lighting it, and of smoking the cigarette on the rate of digital blood flow. The volume (I_V) and rate (I_R) of digital (2RF) blood flow decreased for all subjects when they were offered a cigarette ($p = < .05$). Recovery occurred gradually within a few minutes and the rate of blood flow returned to the control level. However, upon lighting the cigarette, another episode of digital vasoconstriction developed with an associated decrease in the volume and rate of digital flow ($p = < .01$) (Table I and Fig. 1). After recovery was complete, the subject was instructed to smoke a lighted cigarette slowly and to inhale to the same depth as when smoking

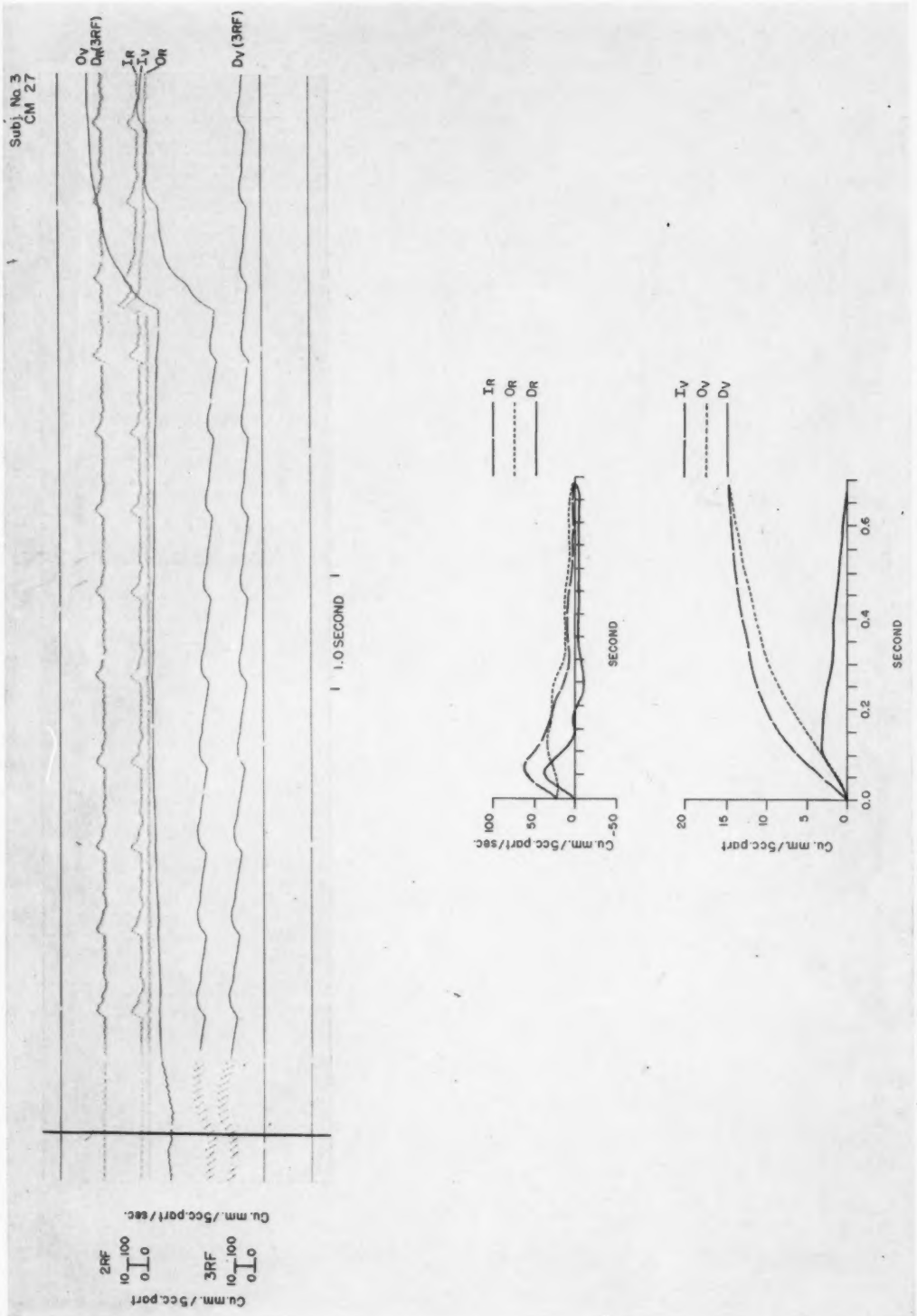

Fig. 4. Digital rheoplethysmorgram for the subject in Figs. 2 and 3. The RPG was recorded just after inhalation of an unlighted cigarette (sham smoking.) Consult legend of Fig. 2 for detailed description of these curves and of the symbols.

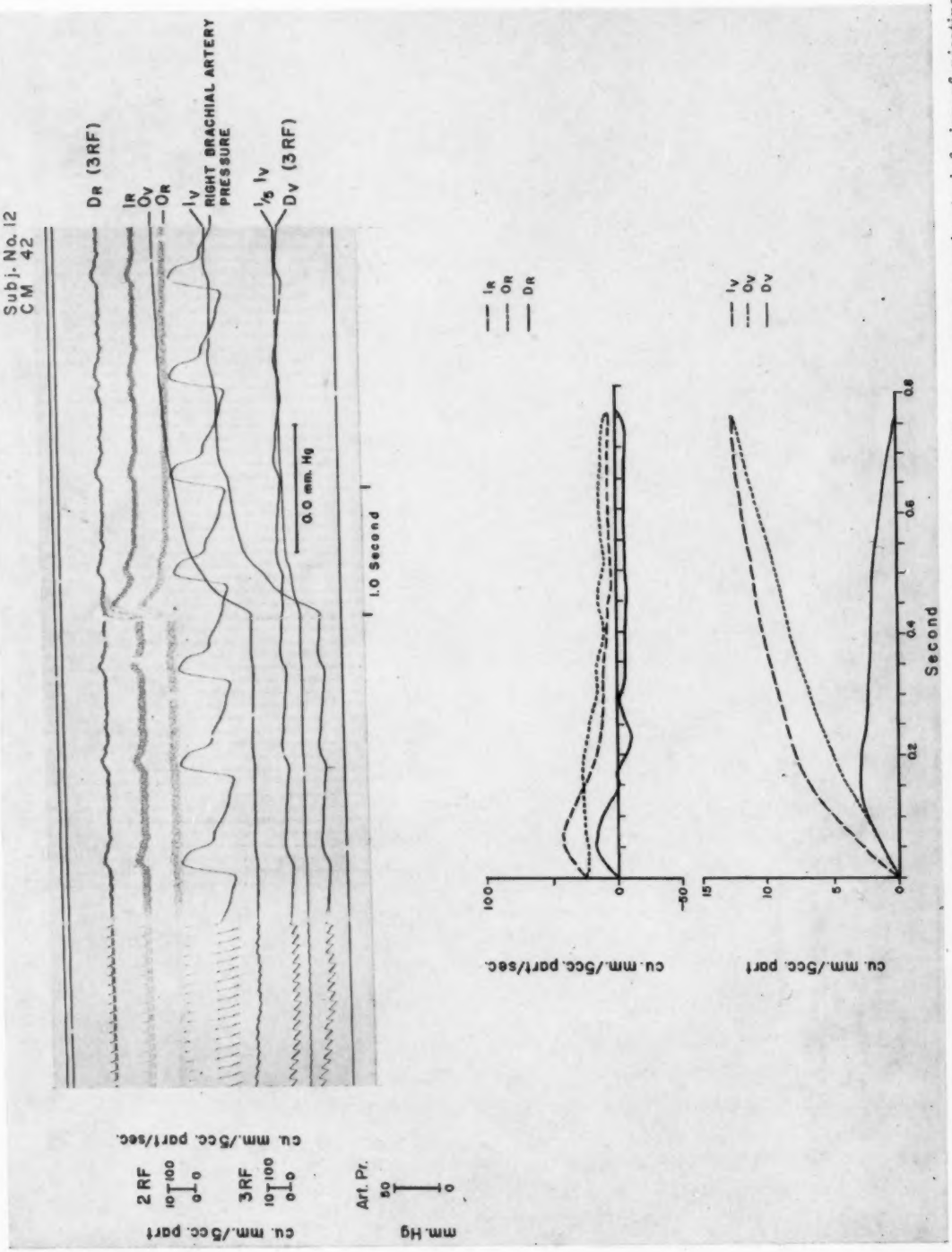

Fig. 5. Subject No. 12. Digital rheoplethysmogram recorded simultaneously with brachial artery pressure prior to infusion of nicotine bitartrate. Consult legend of Fig. 2 for detailed description of these curves and of the symbols.

Fig. 5. Subject No. 12. Digital rheoplethysmogram recorded simultaneously with brachial artery pressure prior to infusion of nicotine bitartrate. Consult legend of Fig. 2 for detailed description of these curves and of the symbols.

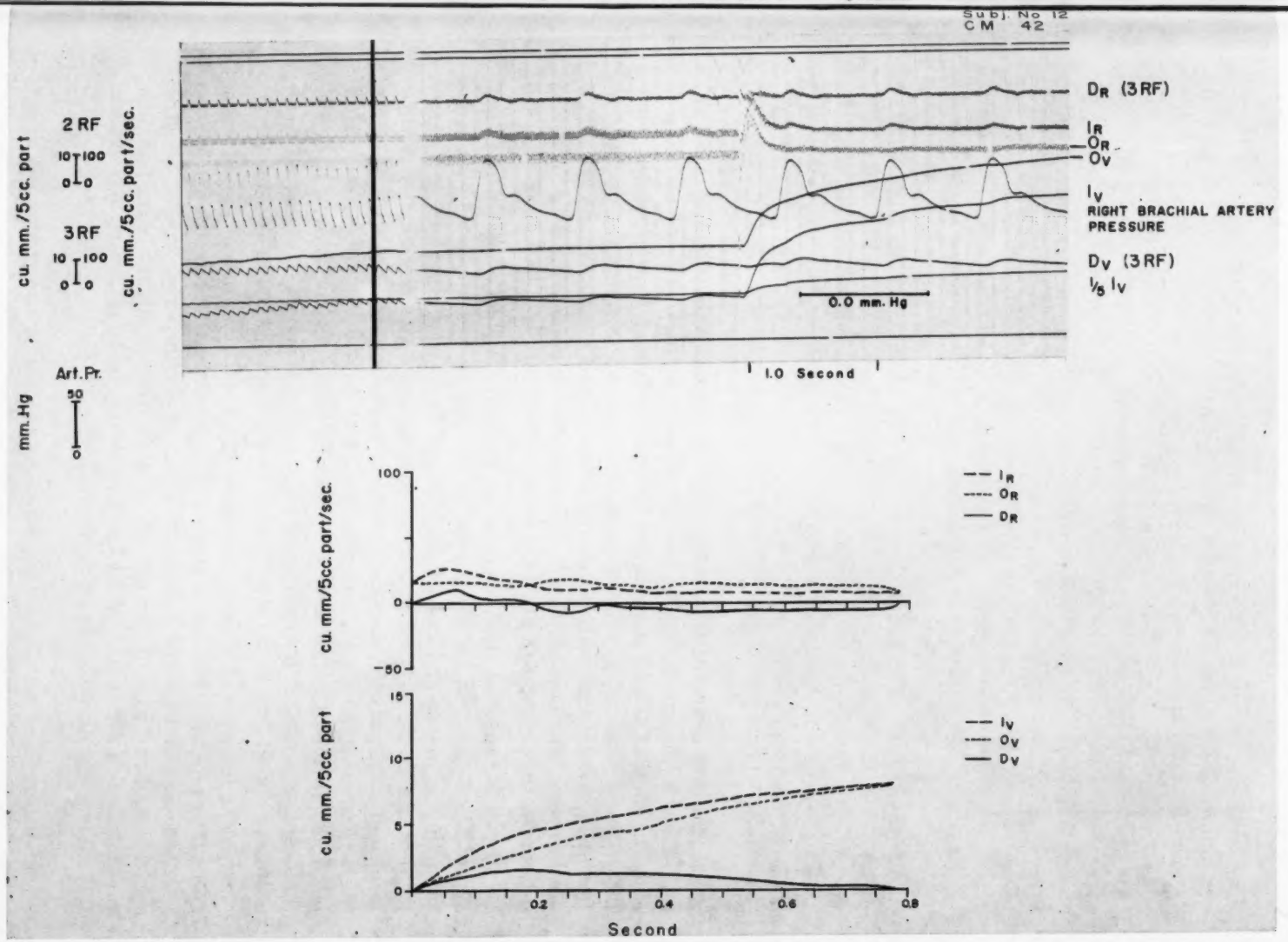

Fig. 6. Digital rheoplethysmogram with simultaneous brachial artery pressure recorded from the same subject shown in Fig. 5 during infusion of nicotine bitartrate. Consult legend of Fig. 2 for detailed description of these curves and of the symbols.

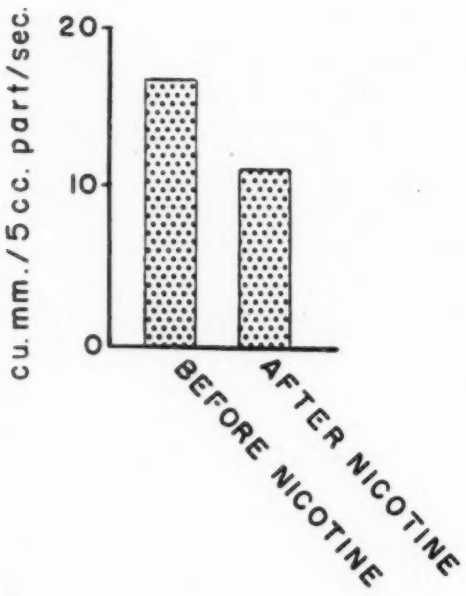

Fig. 7. Influence of nicotine bitartrate on the mean rate of blood flow in 5 subjects.

under ordinary circumstances. With each inhalation of the cigarette there was a decrease in the rate of digital blood flow ($p = < .02$) (Table I and Figs. 2 and 3). In 6 of the 10 subjects the decrease in I_V and I_R was greatest while the cigarette was being lighted, whereas in another subject the decrease in I_V and I_R was greatest when the cigarette was offered to him. In the remaining 3 subjects the maximal decrease in digital blood flow occurred during the actual smoking of the cigarette (Table I).

2. The influence of sham smoking an unlighted cigarette upon the rate of digital blood flow. In all 10 subjects sham smoking was associated with a decrease in the volume and rate of digital blood flow (Figs. 2 and 4). However, the decrease in digital blood flow was not statistically significant ($p = > .10$). In all but 2 subjects the decrease in blood flow was less marked than when the subjects were smoking a lighted cigarette. The decrease in the rate of digital blood flow produced by sham smoking was less marked in all subjects than was the decrease produced by lighting the cigarette (Table I). Deep inspiration decreased digital flow to the same degree as did sham smoking (Table I).

3. The influence of nicotine bitartrate infusion on the rate of digital blood flow. The rapid intravenous infusion of 1 mg. of nicotine bitartrate* resulted in a decrease in both the volume and rate of digital blood flow in all of 5 subjects studied (Figs. 5 through 7). The decrease in digital blood flow was associated with a rise in arterial blood pressure (mean, 22.5 mm. Hg systolic and 8.2 mm. Hg diastolic) in all of the subjects.

*One milligram of nicotine bitartrate was diluted in 50 ml. of normal saline and infused at 45 to 50 drops per minute.

Discussion

Although nicotine produces digital vasoconstriction, experiments designed to study the qualitative or quantitative aspects of the changes in digital blood flow associated with smoking must include a consideration of the vasoconstrictive influences produced by factors other than nicotine itself. It is particularly significant that lighting the cigarette produced a greater decrease in the rate of digital blood flow than did smoking a lighted cigarette. Previous investigations from this laboratory have shown that the digital vascular system is profoundly influenced by psychic factors. For example, an orienting reflex initiated by simple environmental stimuli will produce marked digital vasoconstriction.[4] The utterance of a psychically disturbing word produces marked digital vasoconstriction. It is also possible to condition the digital vascular system to constrict. The vasoconstriction which results from being offered or lighting a cigarette may be, in part, a conditioned reflex, since the anticipation of smoking the cigarette involves certain learned sensations.

The effect of sham smoking and deep inhalation on peripheral blood flow was not as marked as that due to the psychic stimuli already discussed. However, the effect of moderately deep inspiration on the digital circulation cannot be ignored when studying the effect of nicotine in cigarette smoking on digital blood flow.

Many previous experiments in both animals and man have shown that nicotine produces peripheral vasoconstriction. In 5 subjects included in this study the infusion of nicotine bitartrate produced a rapid and marked vasoconstriction. Nevertheless, when measuring the effect of cigarette smoking on the peripheral circulation, the response to multiple stimuli may be involved. In addition to the nicotine in the cigarette, these stimuli may include at least psychic phenomena associated with the act of smoking as well as the vasomotor effects of deep inspiration.

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The intrinsic method for serial plasma volume determination

An analysis of 500 determinations in 76 patients

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An analysis of a large series of serial plasma volume determinations employing the "intrinsic" method is reported. Based as it is on the changes in concentration of substances already in circulation it is extremely simple and widely applicable. No tagged material is necessary after the first determination and a single blood sample yields 2 comparable values. The "extrinsic" method, by contrast, requires injection of tagged material and 2 separate blood samples but yields only one value. The reproducibility of the intrinsic method is indicated by the mean agreement between the 2 values obtained in 479 simultaneous determinations of 100.3 ± 4.2 per cent (S. E., 0.19). In 135 simultaneous determinations by the extrinsic and intrinsic methods the mean agreement between the 2 values was 99.1 ± 7.1 per cent (S. E., 0.59).

Serial blood and plasma volume determinations for study of the shifts of fluid, electrolytes, protein, and cells into and out of the circulation are important in many clinical and laboratory experiments. These include, among others, determining the effect of infusions of crystalloids, colloids, metabolites, or expanders, the testing of anesthetic agents, vasopressors, and sympatholytic agents, and the determination of the effects of environmental changes such as hypo- and hyperthermia. Within recent years sophisticated isotopic methods of plasma, red cell and blood volume determination have been introduced.[4, 5, 8, 19] These, with current modifications and refinements, require complicated, costly instrumentation and highly trained personnel. This has perhaps deterred many investigators from making these important observations.

It is current practice to determine the original plasma volume and the succeeding volumes by the "extrinsic" method. This requires introduction into

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Supported by the Medical Research and Development Board, Office of the Surgeon General, Department of the United States Army, under Contract No. DA-49-007-MD-199.

Received for publication Feb. 13, 1961.

the circulation albumin labeling substances, T-1824,[7] rose bengal, or other dyes,[2] or radioactively tagged native substances (albumin or red cells),[4, 19] and determination of their dilution by the corresponding components in the blood. Serial plasma and red cell volumes are then determined by successive re-application of this procedure. Each determination, however, requires the withdrawal of a blood sample for residual dye or labeled material, the volumetric, gravimetric, or isotopic measurement of the labeling or labeled substance, introduction of the measured material into the circulation, and the withdrawal, after suitable time for mixing, of a second blood specimen. The double sampling, the volume of blood removed, the quantitative measurements and manipulations, the double analyses necessary for each determination, and the accumulation of the labeling material in the blood and tissues impose limitations on both the number of determinations possible and their accuracy.

A much simpler procedure for the determination of serial plasma volumes, after the original has been determined by any of the "extrinsic" methods, depends only on the determination of the changes in concentration of substances already in circulation, i.e., the plasma protein or the red cells, concentration changes in the latter being characterized by both the hemoglobin and the hematocrit. This procedure, to be referred to as the "intrinsic" method, requires only the withdrawal of a single blood specimen and analysis for protein and hemoglobin-hematocrit for 2 simultaneous serial plasma volume determinations.*

The latter method was used for the determination of 7 to 12 serial plasma volumes in each of 76 patients in whom the effects of saline, plasma, and plasma expander infusions were being studied. This experience, and statistical analysis of the data obtained with the method, demonstrate its simplicity, range of usefulness, and reproducibility.

Methods and calculations

Seventy-six hospitalized patients convalescing from injuries or surgical and orthopedic procedures served as the subjects. Lipemic specimens were avoided by keeping the patients on a low fat diet from supper the night before the experiment until it was terminated. All subjects were recumbent for at least ½ hour before blood specimens were drawn. The various experiments from which the data were derived are listed in Table I.

The first blood specimen was drawn and 16.0 mg. of T-1824 (8.0 ml.) injected from a calibrated syringe by the wash-in technique using 10 ml. of saline.[16] Exactly 10 minutes later another specimen was drawn from a vein in the opposite arm and the infusion of 500 or 1,000 ml. of the particular test solution started. Specimens were then drawn 10 minutes and 1, 3, 6, 12, 24, and, in some experiments, 48 or 72 hours after termination of the infusion. Dye then was injected again and a 10 minute specimen drawn for the determination of the 24 (and 48 or 72) hour "extrinsic" dye volume. Thus, each experiment yielded 7 to 12 specimens from which pairs of serial volume values were determined from the changes in both protein and hemoglobin-hematocrit. The values in each pair were then compared to

*Hereafter, the term "volume" will be used to mean plasma volume; volume of red cells and whole blood volume are included by implication, as only the hematocrit is necessary for their calculation. Admittedly, sequestration of cells will lead to a significant difference between whole body hematocrit and peripheral hematocrit and introduce errors in volume calculations. However, since it was shown in these experiments that sequestration does not occur, and since these studies are concerned with changes in volume calculated by a ratio of the hematocrits, it is immaterial whether the ratio used is between peripheral or whole body hematocrits. Similarly, since the trapped plasma will be proportionately the same in each peripheral hematocrit, it will be canceled out in the ratio and a correction for it need not be made.

Table I. The experimental series

Solution infused	*Volume infused (ml.)*	*No. of experiments*	*No. of serial intrinsic volume determinations**
Dextran (Plavolex)	500	5	35
	1,000	6	43
Dextran (Expandex)	500	7	68
	1,000	5	35
Modified fluid gelatin	500	12	81
	1,000	8	55
Oxypolygelatin	500	7	46
	1,000	5	36
Plasma	600	9	63
Saline	1,000	5	36
Dye (T-1824)	(-)	7	61
Total		76	559

*Each volume was determined by *both* the protein and the hemoglobin-hematocrit method except in the plasma experiments where, of course, only the hemoglobin-hematocrit method could be applied. Discrepancies between these figures and succeeding ones are due to this fact, the clotting of a few oxalated specimens, the omission of an occasional midnight specimen, and the refusal of 2 patients to allow completion of the experiments.

each other. Each experiment also yielded a 24 (and 48 or 72) hour extrinsic dye re-injection volume which could be compared with the corresponding two values determined by the intrinsic method on the same blood specimen.*

All blood specimens, 12 ml. each, were drawn without stasis into oiled syringes and partitioned. Three milliliters was added to an appropriate amount of potassium-ammonium oxalate in a test tube for determination of the hemoglobin and hematocrit. Nine milliliters was placed in an oiled test tube and the serum obtained by centrifugation completely cleared, after separation from the clot, by a second centrifugation. The hematocrit was determined in Wintrobe tubes centrifuged at a radius of 15 cm. at 3,000 r.p.m. for at least 45 minutes. The hemoglobin was determined by the alkaline hematin method and the protein by the biuret method. The optical densities of the protein and hemoglobin solutions and of the dyed plasma were determined on the Coleman 6A spectrophotometer. The hematocrits were determined in duplicate in only 18 experiments but the protein and hemoglobin concentrations were determined in duplicate throughout.

The value for the original (dye) volume, PV_o, was calculated from the following relationship: $PV_o = \frac{\text{mg. dye} \times K}{L_o}$, L_o being the optical density of the 10 minute serum specimen, and K the optical density factor for the dye. (This was redetermined for each new lot of dye by serial dilution with serum and determination of 8 to 10 optical density values between the 10 and 90 per cent transmittance range of the spectrophotometer. The average of these values was used as the factor.) The dye re-injection volume was calculated similarly but the optical density of the serum specimen taken immediately prior to the dye re-injection was subtracted from the L_t value to correct for the residual dye in circulation.

The serial volumes were calculated from the protein concentrations (A), and from the

*The calculations of original expansion, changes in expansion with time, loss of expander substance from the circulation and derivative data have been reported and are not germane here.[12-14] This presentation will deal with only the reproducibility of the values obtainable with the two components of the intrinsic method and a comparison of these values with those obtained by the extrinsic method.

hemoglobin-hematocrit concentrations (B), on the basis of the original volumes and the following relationships: the subscript "o" designates the original value, the subscript "t" the value at any given time thereafter, and the letter "s" the correction for the cumulative amount of plasma removed in the specimens up to time "t," which is calculated from the known volume of the blood samples and their corresponding hematocrits:

$$(A)\ PV_t = (PV_o - s) \times \frac{protein_o}{protein_t} \qquad (B)\ PV_t = (PV_o - s) \times \frac{Hgb_o}{(1-Ht_o)} \times \frac{(1-Ht_t)}{Hgb_t}$$

The total circulating protein (T.P.) and the total circulating red cell volume (R.C.V.) were calculated from the relations indicated in (C) and (D):

$$(C)\ T.P. = P.V. \times \text{protein concentration.} \qquad (D)\ R.C.V. = P.V. \times \left(\frac{1}{1-Ht} - 1\right).$$

In the statistical analysis of the paired values the second value was calculated as a ratio of the first in per cent. The means for the sums of the various percentage values and the standard deviations and errors were calculated by the usual formulas. The actual magnitudes of variation in the moieties being measured are derived simply by multiplication of the percentage variation (standard deviation) by the particular base-line values in Table II.

Results

1. Base line values. The values characterizing the group of experimental subjects are given in Table II. The ranges of the values for the plasma, red cell, and whole blood volumes are rather wide but are consistent with the wide range in the weights of these patients. The mean values are slightly lower than those given for healthy young adults, but many of the subjects were middle-aged or older, had sustained trauma or been operated upon recently, and 8 or 10 were moderately anemic or hypoproteinemic. These values, however, are practically identical to those recently reported in a collected series of 571 patients,[15] which were 43.9, 32.9, and 77.1 ml. per kilogram, as compared to the values in Table II of 43.9, 31.3, and 74.0, respectively.

2. Reproducibility of the analyses and of concentration and volume determinations. The reproducibility of the analyses, of the concentration determina-

Table II. Base line values

	Range	*Mean*	*Standard deviation*	*Standard error*
Weight (Kg.)	35.4 -100.0	71.1	17.2	1.72
Hemoglobin (Gm. %)	6.99- 15.65	12.93	1.60	0.18
Hematocrit (%)	26.3 - 51.7	43.1	5.1	0.58
Protein (Gm. %)	4.80- 7.90	6.71	0.59	0.06
Plasma volume (ml.)	1,807-5,915	3,005	628	71
Red cell volume (ml.)	1,148-3,805	2,306	530	61
Blood volume (ml.)	3,200-9,200	5,275	1,037	119
Plasma volume (ml./Kg.)	27.4 - 65.1	43.9	8.60	1.00
Red cell volume (ml./Kg.)	18.1 - 59.4	31.3	5.44	0.67
Blood volume (ml./Kg.)	50.6 - 99.1	74.0	11.64	1.42

tions, and of the volume determinations is indicated in Table III. The degree of agreement between the duplicate analyses for hemoglobin, hematocrit, and protein are indicated in section *A* of the table. The reproducibility of the determinations of the concentrations and the corresponding volumes calculated therefrom are indicated in Table III, section *B*. Here the values for a given specimen were compared with those obtained 10 minutes later (the pre- and post-dye injection specimens taken 10 minutes apart at the start and at the termination of the experiments). The variations occurring after a 24 hour period in the concentrations and volumes in the control experiments are indicated in section *C* of the table, where the 24 hour values were compared to the original values.

From Table III, section *A*, it is seen that the analyses have a variation of ± 1 per cent. The procedural manipulations (venipunctures, partition of the blood sample, separation of the serum, etc.) and the intravascular variations add another 1 per cent so that 10 minute specimens (section *B*) vary by ± 2 per cent. The spontaneous variations over 24 hours add still another 1 to 2 per cent so that daily specimens (section *C*) vary by ± 3 to 4 per cent. The protein volumes, as anticipated, have practically the same variation as do the protein concentrations. The hemoglobin-hematocrit 10 minute and 24 hour volumes, however, vary somewhat more than the corresponding concentration determinations. This is to be expected, since the latter volumes are derived from 2 variables and the former from only one. The dye determined (re-injection) volume varied from the original by ± 5.8 per cent. Here the greater range of

Table III. Reproducibility of the analyses, concentration determinations, and volume determinations

	A *Duplicate analyses*				*B* *Interval determinations (10 minute)**				*C* *Interval determinations (24 hour)†*			
	No. of values	*% Mean*	*% S. D.*	*% S. E.*	*No. of values*	*% Mean*	*% S. D.*	*% S. E.*	*No. of values*	*% Mean*	*% S. D.*	*% S. E.*
Hemoglobin	686	100.0	0.88	0.034	157	100.1	1.89	0.15	100	100.3	3.89	0.39
Hematocrit	130	100.2	0.88	0.077	156	100.3	1.96	0.16	100	100.2	3.34	0.33
Protein	703	100.2	1.33	0.050	159	99.6	2.27	0.18	98	100.7	3.05	0.31
Protein volume					68	99.5	2.10	0.26	95	99.3	3.08	0.32
Hemoglobin-hematocrit volume					74	99.8	2.50	0.29	94	99.4	5.53	0.57
Dye volume									16	99.0	5.81	1.45

*This is the comparison between the values on the specimens taken 10 minutes apart at the original volume determination plus those taken 10 minutes apart for the dye volume redetermination 24 hours later.

†This group indicates the reproducibility of concentration and volume values with an interval of 24 hours in the control experiments receiving only dye or saline but no expander solution. This group includes the 5 saline and 7 dye experiments, plus data from another series, in which determinations were made daily for 3 to 14 days in following the time course of disappearance of T-1824.

variation is no doubt due in part to the inaccuracies in the measurement of the dye and its injection and in part to the double sampling and dye readings necessary for the final determination.

3. Comparison of the volumes determined by protein, hemoglobin-hematocrit, and dye. The agreement between the volumes determined by the two intrinsic methods and between these and the dye re-injection volumes at 24 hours is indicated in Table IV. The variation between the individual hemoglobin-hematocrit volumes and the corresponding protein volumes for 479 determinations was ± 4.2 per cent. The variation between these and the dye re-injection volumes at 24 hours was ± 6.7 per cent for the hemoglobin-hematocrit and ± 7.5 per cent for the protein volumes.

The gradual but very slight increase in difference between the two intrinsic volumes over a 24 hour period is indicated in Table V. The variation between the two is 3.75 per cent up to 3 hours, 4.15 per cent between 6 and 12 hours, and 4.85 per cent at 24 hours. The constancy of the physiologic factors being measured and the reproducibility of the analyses is indicated in the increase by only 1.1 per cent in the difference between the two volumes over 24 hours. Thus, over a 24 hour period, serial volumes determined by both the intrinsic methods may be expected to agree with each other within an average of ± 4.2 per cent. In actual units this would imply a variation of ± 127 ml. from the mean plasma volume of 3,000 ml. and a variation of ± 98 ml. from the mean red cell volume of 2,300 ml.

4. Total protein, total red cell volume, and hemoglobin-hematocrit ratio. The application of both of the intrinsic methods to the determination of serial volumes in these experiments is predicated on two assumptions. These are that neither red cells nor plasma proteins are sequestered in or displaced from the circulation by the sudden increase in plasma volume or the addition of the osmotically active expanders. The close agreement between the protein and hemoglobin-hematocrit volumes would seem to support this assumption; it is hardly likely that both protein and red cells would be displaced in the same proportion to give the close agreement between the two. Although reported studies[12, 18] also support these assumptions, they were specifically tested as follows: The total protein in circulation at the various intervals following the infusion in each experiment was determined from the product of the *hemoglobin-hematocrit* volume and the corresponding protein concentration and calculated as a percentage of the original pre-infusion total protein. Similarly, the total volume of red cells in circulation was determined from the *protein* volume and the hematocrit at each interval and calculated as a percentage of the original (pre-infusion) total red cell volume. Although the formula for volume by the hemoglobin-hematocrit method (equation B, above) automatically corrects for any change in hematocrit due to swelling or shrinkage of the red cells should such occur, the exact degree of change was calculated. The hemoglobin to hematocrit ratios at the various intervals after the infusion were calculated as a percentage of the original pre-infusion ratio. Since total hemoglobin remains constant, shrinkage of the cells would result in a lower hematocrit and a corresponding rise in

Table IV. Comparison of hemoglobin-hematocrit volumes with protein volumes and of both of these with dye re-injection volumes

	No. of values	*% Mean*	*% S. D.*	*% S. E.*
Hemoglobin-hematocrit versus protein volumes	479	100.3	4.24	0.19
Hemoglobin-hematocrit versus dye re-injection volumes	142	98.9	6.70	0.56
Protein versus dye re-injection volumes	127	99.3	7.47	0.61

Table V. Comparison of hemoglobin-hematocrit volumes with protein volumes at progressive intervals

Time interval after infusion	*No. of values*	*% Mean*	*% S. D.*	*% S. E.*
10 minutes	59	102.4	3.86	0.50
1 hour	65	100.3	3.66	0.45
3 hours	65	99.6	3.72	0.46
6 hours	64	100.2	4.17	0.52
12 hours	61	101.1	4.10	0.53
24 hours	64	100.1	4.96	0.62
24 hours 10 minutes	65	100.3	4.75	0.59
Total or mean	443	100.5	4.24	0.20

Table VI. Variation in total protein, red cell volume, and hemoglobin-hematocrit ratio

	No. of values	*% Mean*	*% S. D.*	*% S. E.*
Total protein	484	101.0	4.30	0.20
Total red cell volume	488	99.4	4.39	0.20
Hemoglobin-hematocrit ratio	542	100.8	1.87	0.08

the ratio; conversely, swelling of the cells would increase the hematocrit and lower the ratio. The validation of the assumptions and the absence of any significant change in red cell size are indicated by the results in Table VI.

The variations in total protein and total red cell volume are in the range expected from the variation in the concentration determinations from which they were calculated and do not seem to indicate any major sequestration in or displacement from the circulation. The range in variation of the hemoglobin-hematocrit ratio is what would be expected from the range of variation in the determination of its two moieties and the magnitude of the variation would indicate no significant change in red cell size as a result of the infusion of saline, expander, or plasma. This is further confirmed by the fact that the

variation for 70 values in the control series (where only dye was injected and serial samples were taken) was practically the same; the mean, S. D., and S. E. were 100.0 ± 1.48 and ± 0.18, respectively.

Discussion

The above data indicate that the determinations of serial plasma volumes up to 24 hours or longer by the "intrinsic" methods are reproducible within a very acceptable range of variation for many clinical and experimental situations. This method is of course *not* applicable where plasma and red cells are being lost simultaneously and in unknown proportions, such as in crushing injury or bleeding. In such situations, during periods of arrest of the bleeding or other losses, serial use of the extrinsic method is necessary and will give information not obtainable with the intrinsic method. The latter is, however, applicable in all situations where plasma water or extracellular fluid is being added to, or lost from, the circulation, and determination of both the hemoglobin-hematocrit and protein concentration changes will permit calculation of the volume changes. If plasma water and protein in unknown proportions are being lost from the circulation, as in rapid effusions, peritonitis, or second-degree burns, determination of the changes in hemoglobin-hematocrit will allow calculation of the changes in circulating plasma volume and determination of the plasma protein concentration will allow calculation of the amount of protein lost. Conversely, if protein-containing solutions such as albumin or plasma are infused, the changes in circulating plasma volume and total protein can be determined similarly. Even when both plasma and red cells are being lost from the circulation, but in measurable volumes, as in experimental hemorrhage, the losses of protein and hemoglobin and the shifts of fluid into or out of the circulation may be calculated quite accurately from the values of the hemoglobin-hematocrit and protein before the bleeding, the values on the shed blood, and on the blood remaining in circulation.

The identical nature of the various methods utilizing the extra- or intravascular tagging of albumin with dye or isotope for the extrinsic determination of plasma volume has been amply demonstrated.[2, 6, 9, 17] It is therefore immaterial whether T-1824, rose bengal, or I^{131}-labeled albumin is used to determine an original or re-injection volume. The much closer agreement in these experiments of the hemoglobin-hematocrit volumes with the protein volumes than either of these with the dye re-injection volumes (Table IV) suggests the superiority of the two intrinsic methods to the extrinsic re-injection method for serial volume determination. They are certainly preferable from the technical aspect. Simply by determining hemoglobin, hematocrit, and protein, two values for plasma volume are derived which can be compared with each other and averaged for a relatively precise value.

A semimicromodification of the method described may be suggested. Much smaller samples may be drawn to obviate sampling corrections and to avoid fluid shifts by removing large volumes as samples. Only 1.5 ml. of blood is necessary for duplicate determinations of hemoglobin, hematocrit, and protein.

The hemoglobin determinations require only 40 cu. mm. of blood when a Sahli pipette is used. After centrifugation and reading of the hematocrit the supernatant may be aspirated and used for the determination of the plasma protein. This requires only 0.20 ml. for duplicate determination by the biuret method and much less by the falling drop method. Thus, to make 50 or more serial volume determinations over a 24 hour period, or even over a 1 hour period if required by the experimental design, only 75 ml. of blood would need be removed.

Doubt has been cast on the validity of using changes in hematocrit to calculate fluid shifts into and out of the circulation. One observer reported that there was "an apparent loss of red cell mass in patients with the nephrotic syndrome following intravenous administration of human serum albumin where the red cell mass is calculated from the hematocrit and plasma volume." He concluded that "calculations of fluid shifts based on changes of hematocrit values are likely to be seriously in error; such calculations should be avoided."[3] It is quite probable that the "apparent loss of red cell mass" was entirely a result of the shrinkage of the red cells (and falsely low hematocrit) due to the hyperosmotic effect of normal serum albumin in nephrotic patients with severe hypoproteinemia. Determination of the hemoglobin-hematocrit ratio before and after the infusion would probably have demonstrated this shrinkage and correction of the hematocrit for this factor would have eliminated the inaccuracies in the fluid shift calculations. This postulated shrinkage of red cells was actually observed when concentrated albumin was given to normo- and hypoproteinemic dogs.[11] The hemoglobin-hematocrit ratio was raised by 5 to 20 per cent, and calculation indicates that shrinkages of these magnitudes in the hematocrit introduce errors of 10 to 30 per cent in the calculations of plasma volume.

Another observer compared the plasma volume values obtained by dye injection and by changes in hematocrit and protein. He concluded that "although the change in hematocrit value indicated the directional change in plasma volume in every instance it failed to determine quantitative change accurately. The change in plasma protein concentration ... seemed to indicate the degree of plasma volume change more accurately than the hematocrit. However, the agreement was not sufficiently close to warrant quantitative estimation."[10] Review of these experiments indicates that drastic changes were produced in body hydration—severe dehydration and severe over-hydration. No doubt here, too, there were commensurate changes in the size of the red cells and the resulting hematocrits were not truly representative of the proportion of cells in the blood with consequent false calculation of the plasma volumes. Had hemoglobin determinations been carried out simultaneously with the hematocrits the expansion or shrinkage of the cells could have been detected and correct calculations of plasma volume made.

Various modifications in the now standard isotope dilution methods have been proposed recently for the determination of serial volumes. In one, a measured quantity of I^{131}-labeled human albumin is introduced into the cir-

culation before the experiment and the control slope of the disappearing isotope is established. Subsequent deviations from the anticipated points on the curve projected into the experimental period are taken to indicate the degree of dilution or concentration and the corresponding changes in volume.[1] Since the circulating plasma protein has a stable control level, deviations from this level itself during the experimental period will indicate equally well the changes in concentration and volume. Should the experimental conditions be such that normal circulating protein is being lost from the capillary bed, isotope or otherwise tagged albumin would be lost also and neither method would be applicable. Under these conditions, as already indicated above, hemoglobin-hematocrit changes still would allow calculation of changes in volume.

In another method for serial volume determination recently reported, a mean curve of disappearance of radioiodinated albumin is established in a group of control dogs. Deviations in concentration of the tagged material from this mean "predicted disappearance curve" in another group, the experimental animals, are then used as the basis for the calculation of changes in volume.[20] This method is quite indirect and appears to be unnecessarily complicated and laborious. Here, too, the same information regarding changes in volume under the given experimental conditions could have been obtained by determination of protein and hemoglobin-hematocrit changes, with the experimental animals serving as their own controls.

It is no doubt true that single volume determinations by the extrinsic method are required more frequently, particularly in clinical circumstances, than are serial determinations. However, when the latter are indicated in the clinical, experimental, or laboratory situations noted above, the extrinsic dye or isotope methods need not necessarily be used. The intrinsic method, based as it is on the proportional relations of the volume and concentration factors of substances already in circulation, is widely applicable. It fails only when *both* plasma protein and red cells are being lost from the circulation simultaneously and in unknown amounts. In that case, these substances would be lost from the circulation even if tagged, and neither the extrinsic nor the intrinsic method would be of value. In all other circumstances, however, the latter, being reproducible and requiring only one blood sample and the simplest of laboratory equipment and techniques, has certain advantages over the extrinsic dye or isotope method for the determination of serial plasma volumes.

The studies upon which this paper is based were performed in the Surgical Research Laboratories of St. Vincent's Hospital, New York, with the constant encouragement and support of Dr. L. M. Rousselot. The participation of Drs. F. E. Gilbertson and J. M. Harmon in the performance of some of the experiments during the trimester they each spent in the laboratory is greatly appreciated. The technical assistance of Anne Siket and Sally Davis is gratefully acknowledged.

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The hemolytic effect of primaquine. XIV. Pentose metabolism in primaquine-sensitive erythrocytes

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The oxidative decarboxylation of glucose-6-phosphate, initiated by glucose-6-phosphate dehydrogenase in the pentose phosphate pathway, results in the regeneration of reduced triphosphopyridine nucleotide and the formation of CO_2 and pentose phosphate. Although deficient activity of the enzyme glucose-6-phosphate dehydrogenase in primaquine-sensitive erythrocytes impairs the regeneration of triphosphopyridine nucleotide and the production of CO_2, the total pentose content was found to be equal in sensitive and normal erythrocytes. In addition, the total capacity for the reversible conversion of pentose phosphate to triose phosphate and hexosemonophosphate in hemolysates by the transaldolase and transketolase exchange reactions of the pentose phosphate pathway was found to be equal in sensitive and normal erythrocytes.

Acute intravascular hemolysis occurs in approximately 10 to 15 per cent of American Negroes,[1] in higher percentages of non-Ashkenazie Jews[2] and Sardinians,[3] and in varying percentages of other widespread groups[4] following the ingestion of many compounds, including primaquine,[1] several nitrofurans,[5, 6] certain sulfonamides,[7] and fava beans.[8] Susceptibility to this chemically induced hemolysis is genetically determined by a sex-linked gene of intermediate dominance.[9-14]

From the University of Chicago-Army Medical Research Unit, Chicago, Ill. Supported in major part by the Research and Development Command, Office of the Surgeon General, Department of the Army, under Contracts Nos. DA-49-007-MD-566 and DA-49-117-MD-968 with the Department of Medicine, University of Chicago, Chicago, Ill.; and in part by Winthrop Laboratories, Inc., New York, N. Y.; Parke, Davis & Co., Detroit, Mich.; and Eaton Laboratories, Inc., Norwich, N. Y.

The investigations reported in this paper would not have been possible without the cooperation of the inmates and administrative staffs of the Illinois State Penitentiary, Stateville Branch, Joliet, Ill.

Received for publication Nov. 28, 1960.

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Erythrocytes of primaquine-sensitive Negroes demonstrate several biochemical abnormalities, including a deficiency of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD*),[15] diminished GSH content,[16] and diminished catalase activity[17] (decrease further during in vivo primaquine-induced hemolysis[17, 18]) and an increase in glutathione reductase activity[19] and aldolase activity.[20] The most prominent defect is the deficiency of G-6-PD activity, this enzyme catalyzes the first oxidative reaction of the pentose phosphate pathway (PPP). The G-6-PD deficiency impairs the ability of primaquine-sensitive erythrocytes to reduce TPN[6, 21] and to decarboxylate G-6-P under physiologic conditions.[22] This defect in the oxidative portion of the PPP might also diminish both the formation and the content of pentose phosphate in primaquine-sensitive erythrocytes. Pentose phosphate can be derived from either the oxidative decarboxylation of G-6-P and 6-phosphogluconic acid, the transketolase and transaldolase exchange reactions of the nonoxidative portion of the PPP (Fig. 1), or the breakdown of nucleosides.[23-25] The data in this report compare primaquine-sensitive† and normal erythrocytes with respect to (1) the over-all activity of the nonoxidative reactions of the PPP, (2) changes in total pentose content before and after incubation with glucose in vitro, and (3) oxygen uptake by hemolysates and intact erythrocytes.

Materials

Barium R-5-P‡ was converted to the sodium salt and neutralized before use. This preparation contained no detectable hexosemonophosphate or hexosediphosphate esters and only trace amounts of triose phosphate by enzymatic assay; stock solutions (0.05 M) were prepared and stored at −20° C. The pyridine nucleotides DPN^+ and TPN^+§ were prepared as aqueous solutions; their purity was determined enzymatically, and they were stored at −20° C. Thiamine pyrophosphate‖ was prepared fresh in aqueous solution (2.4×10^{-3} M). G-6-PD# was prepared fresh at the time of use as an aqueous solution, approximately 0.6 Kornberg units per milliliter. Glyceraldehyde phosphate dehydrogenase and triose isomerase were prepared fresh weekly from human erythrocytes, as previously described.[20] Stroma-free, dialyzed, and Norit treated hemolysate was prepared from human erythrocytes as described by Schrier and co-authors.[20] This hemolysate was free of interfering amounts of GSSG, GSH, and pyridine nucleotides.

Methods

1. Reactions using hemolysates.

A. CONVERSION OF R-5-P TO TRIOSE PHOSPHATES AND HEXOSEMONOPHOSPHATES. In a final volume of 7 ml., R-5-P (25 μmoles) was incubated aerobically at 37° C. with 2 ml. of

*Abbreviations in this paper will be as follows: DPN^+, diphosphopyridine nucleotide; G-6-P, glucose-6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; GSH and GSSG, reduced and oxidized glutathione, respectively; HP, hexosemonophosphate; PKPE, phosphoketopentoepimerase; PPP, pentose phosphate pathway or hexosemonophosphate shunt; PRI, phosphoriboisomerase; R-5-P, ribose-5-phosphate; TA, transaldolase; TK, transketolase; TP, triose phosphate; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively.

†Primaquine-sensitivity or nonsensitivity was ascertained in all cases by measurement of G-6-PD activity, the glutathione stability test, and by administration of 30 mg. primaquine base daily for 14 days. None of the volunteers had received hemolytic compounds for at least 4 months prior to their participation in this study.

‡Nutritional Biochemicals Corp., Cleveland, Ohio.

§Sigma Chemical Co., St. Louis, Mo.

‖Cocarboxylase, Schwarz Laboratories, Inc., Mt. Vernon, N. Y.

#Sigma Practical Type II, Lot No. 18-136-3, 1,500 Kornberg units per gram, Sigma Chemical Co., Minneapolis, Minn.

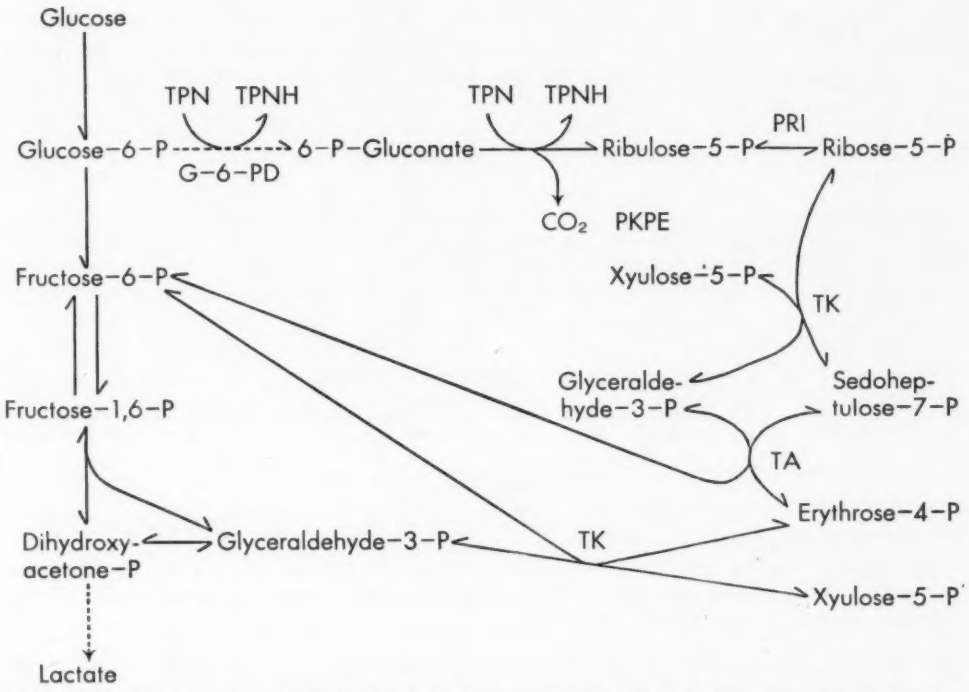

Fig. 1. Schematic representation of carbohydrate metabolism in the human erythrocyte. The reversible transaldolase and transketolase exchange reactions of the pentose phosphate pathway are on the right. The net result of a theoretic summation of the transketolase and transaldolase reactions would result in 2 moles of fructose-6-phosphate and 1 mole of trios phosphate from 3 moles of pentose phosphate.

hemolysate (adjusted to 5 Gm. per cent hemoglobin), thiamine pyrophosphate (2.4 μmoles), and $MgCl_2$ (10 μmoles). After zero, 1, 2, 3, or 4 hours of incubation, the reaction was stopped by the addition of 5 ml. of 4.5 per cent metaphosphoric acid and the contents were swirled for 5 minutes and then filtered. The zero hour reaction contained all reagents, but the metaphosphoric acid was added before the hemolysate. Eight milliliters of each filtrate was neutralized, completed to a 10 ml. volume, and assayed enzymatically for TP and HP content.

B. ASSAY FOR HEXOSEMONOPHOSPHATE. One milliliter of the filtrate from A, above was incubated at 20° C. with 250 μmoles TRIS, pH 7.4, 3 μmoles TPN^+, and approximately 0.6 Kornberg units G-6-PD in a final volume of 3 ml. in a cuvette (1 cm. light path). The reduction of TPN^+ and DPN^+ was measured by the increase in absorption at 340 mμ (extinction coefficient $= 6.22 \times 10^6$ cm.^{2} per mole)[26] in a Beckman DU spectrophotometer with a photomultiplier attachment. The G-6-PD preparation contained an active phosphohexose isomerase but no aldolase and was not inhibited by neutralized metaphosphate in the concentration used in this study.

C. ASSAY FOR TRIOSEPHOSPHATE. One milliliter of the filtrate from A, above was incubated at 20° C. aerobically with 500 μmoles TRIS buffer, pH 8.4, 50 μmoles GSH, 44 μmoles sodium arsenate, 17.4 μmoles DPN^+, and an excess of glyceraldehyde phosphate dehydrogenase and triose isomerase in a final volume of 5 ml. Maximal reduction of DPN^+ occurred after 90 minutes, of reaction.

D. OXYGEN CONSUMPTION BY HEMOLYSATES. The oxygen consumption by hemolysates was determined in a Warburg manometer. Erythrocytes were washed once in 0.145 M NaCl and resuspended in an equal volume of 0.145 M NaCl. Two milliliters of this erythrocyte suspension were incubated in air at 37° C. with 100 μmoles phosphate buffer, pH 7.4, 30 μmoles

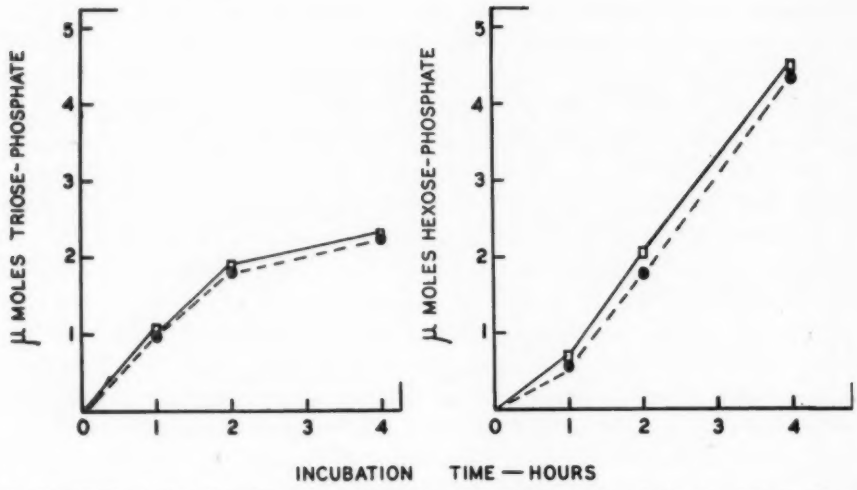

Fig. 2. Atypical study showing the conversion of ribose-5-phosphate to triose phosphate and hexosemonophosphate by hemolysate from a primaquine-sensitive (●----●) and a normal Negro man (□—□) per unit time. The over-all activity of this enzymatic conversion was calculated as micromoles of triose phosphate or hexosemonophosphate formed per unit time per 10 mg. of hemolysate hemoglobin.

glucose-6-phosphate, 1.34 μmoles TPN^+, 134 μmoles methylene blue, and H_2O to make a final reaction volume of 7.3 ml. The above reaction also was done without methylene blue or additional TPN^+ (experiment of E. S. G. Barron).

2. Reactions using intact erythrocytes.

A. OXYGEN UPTAKE BY INTACT ERYTHROCYTES. The oxygen uptake by intact erythrocytes was determined with and without the addition of methylene blue using a Warburg manometer. Two milliliters of packed erythrocytes (washed with 0.145 M NaCl) were incubated at 37° C. with 20 μmoles phosphate buffer, pH 7.4, 40 μmoles glucose, and 0.5 μmoles of methylene blue in air in a final volume of 2.55 ml. A similar reaction mixture was made without methylene blue or additional TPN^+ (experiment of E. S. G. Barron).

B. DETERMINATION OF TOTAL PENTOSE. The total pentose content of erythrocytes was determined by the method of Mejbaum[27] as modified by Barron. Equal volumes of the specimen to be determined (whole blood, plasma, or erythrocyte suspension) and 10 per cent trichloroacetic acid were mixed. After 30 minutes the precipitate was removed by centrifuging (> 6,000g) or filtering. Aliquots of supernatant containing no more than 0.6 μmoles pentose were used for analysis. The reaction mixtures contained 2.0 ml. of aqueous 1.2×10^{-3} M orcinol,* 8.0 ml. of 7 by 10^{-2} M $FeCl_3$ in concentrated HCl, the aliquot for determination, and H_2O to a final volume of 16.0 ml. The mixtures were boiled for exactly 40 minutes in tubes covered with glass marbles and then cooled by placing in cold water. The color was allowed to stabilize for 60 minutes and absorbancy (O. D.) determined at 670 and 580 mμ in a Beckman B spectrophotometer using cylindrical cuvettes of 25 mm. light path. Readings were done at 60 minutes after boiling, although the developed color was stable overnight. Total pentose was calculated by the formula: $(3.5\ O.D._{670} - O.D._{580})\ (0.094) =$ micromols per 16 ml. reaction mixture.

These conditions were checked by obtaining control spectra in the Cary Model 14 recording spectrophotometer. There was no color development with orcinol alone or with 6-phosphogluconate, fructose-6-phosphate, and fructose-1, 6-diphosphate. The method does not distinguish free ribose from phosphorylated pentoses either free or occurring as part of purine nuleosides.

*Lot No. 46, Eastman Organic Chemical Co., Rochester, N. Y.

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C. ERYTHROCYTE PENTOSE FORMATION. Pentose formation by intact erythrocytes was determined with and without the addition of methylene blue. Two milliliters of erythrocytes, washed 3 times with 0.145 M NaCl, was incubated at 37° C. with 20 μmoles phosphate buffer, pH 7.4, 40 μmoles glucose, and 0.5 μmoles of methylene blue in a final volume of 2.55 ml. Incubations were performed aerobically. After 180 minutes, 2 ml. of this suspension were mixed with 2 ml. of 10 per cent trichloracetic acid, and pentose content was determined on the filtrate.

D. EFFECT OF PRIMAQUINE AND "PRIMAQUINE PLASMA" ON THE NONOXIDATIVE PORTION OF THE PENTOSE PHOSPHATE PATHWAY. Primaquine-sensitive and nonsensitive erythrocytes were incubated at 37° C. with (1) plasma obtained from a compatible donor who had received 240 mg. of primaquine base in divided doses during the previous 24 hours, (2) plasma to which was added 0.023 μmoles of primaquine base per milliliter, or (3) appropriate plasma controls; the adjusted hematocrit was 47 per cent. Following a 4 hour incubation, the cells were washed 3 times with 3 volumes of 0.145 M NaCl and converted to hemolysate. The ability of hemolysates of these erythrocytes to convert R-5-P to TP and HP was assayed as described.

Results

1. Reactions using hemolysates.

A. CONVERSION OF R-5-P TO TRIOSE PHOSPHATE AND HEXOSE MONOPHOSPHATE. Hemolysates from primaquine-sensitive and nonsensitive individuals formed TP and HP equally well after ½, 1, 2, 3, and 4 hours of incubation (Fig. 2) and with various hemolysate concentrations (Fig. 3). The ratio of HP to TP increased as the reaction time increased; after 4 hours the ratio was usually 2:1 (HP:TP). These results are in agreement with those of Dische[28] (although the methods of assay are different) and are consistent with the summation of the nonoxidative reactions of the PPP (Fig. 1).

B. OXYGEN CONSUMPTION BY HEMOLYSATES. Hemolysates of nonsensitive erythrocytes utilize more oxygen than hemolysates of primaquine-sensitive

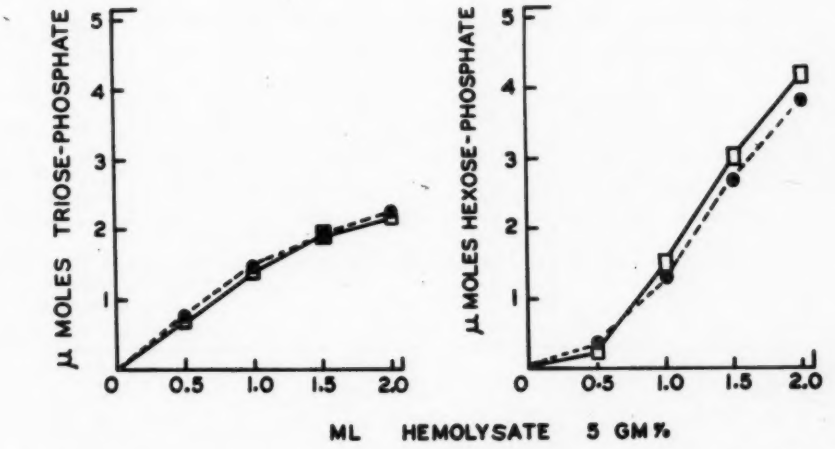

Fig. 3. Triose phosphate and hexosmonophosphate production from ribose-5-phosphate by increasing increments of hemolysate from a primaquine-sensitive (□—□) and a nonsensitive (●---●) Negro man.

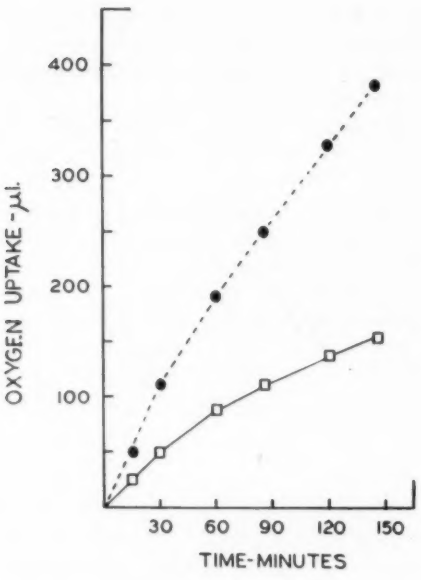

Fig. 4. A representative study showing the decreased oxygen uptake by intact erythrocytes (in the presence of methylene blue) from a primaquine-sensitive Negro man. (□—□, primaquine-sensitive man; ●---●, nonsensitive man.) These data were obtained from E. S. G. Barron.

erythrocytes in both the presence and in the absence of methylene blue and additional TPN^+ (Table I). Oxygen uptake was 6 to 10 times less when TPN^+ and methylene blue were omitted.

2. Reactions using intact erythrocytes.

A. OXYGEN CONSUMPTION BY INTACT ERYTHROCYTES. In the presence of methylene blue oxygen utilization by sensitive erythrocytes was consistently less than the uptake of nonsensitive erythrocytes (Fig. 4). Johnson and Marks[30] also have reported diminished oxygen utilization by primaquine-sensitive erythrocytes in the presence of methylene blue.

B. ERYTHROCYTE PENTOSE CONTENT. The average total pentose content of primaquine-sensitive erythrocytes was 232 µmoles per 100 ml. erythrocytes (range, 209 to 253 µmoles, 9 individuals), and the average total pentose content

Table I. The complete system used for the determination of oxygen uptake contained lysed erythrocytes, phosphate buffer, glucose-6-phosphate, $MgCl_2$, TPN^+ and methylene blue (these data were collected by E. S. G. Barron)

Subjects	*Oxygen uptake microliters per minute per 10^{10} R.B.C. (lysed)*	
	Complete system	*Methylene blue and TPN omitted*
Nonsensitive men		
No. 1	4.74	—
No. 2	4.80	.77
Sensitive men		
No. 1	3.74	—
No. 2	3.58	.34

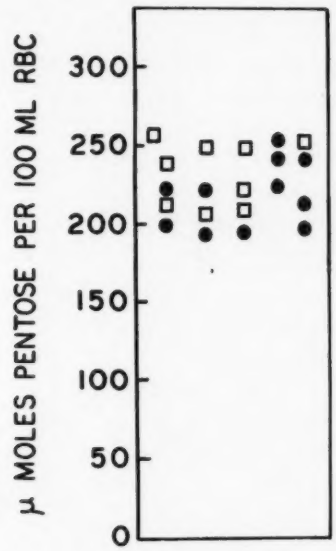

Fig. 5. Total pentose in erythrocytes from primaquine-sensitive (□) and normal Negro men (●).

of nonsensitive erythrocytes was 218 μmoles per 100 ml. of erythrocytes (range, 192 to 253 μmoles, 11 individuals) (Fig. 5). Brunetti and Broccia[29] also have found that the total pentose content is equal in sensitive and normal erythrocytes.

C. PENTOSE FORMATION. The data in Table II show the net accumulation of pentose by intact erythrocytes in the presence of methylene blue relative to that formed by erythrocytes without methylene blue. The erythrocytes of nonsensitive men accumulated more than 1 μmoles in 3 hours but the erythrocytes of sensitive men showed no pentose accumulation after 3 hours.

D. INCUBATION OF ERYTHROCYTES WITH "PRIMAQUINE PLASMA" OR PLASMA TO WHICH PRIMAQUINE POWDER WAS ADDED. The conversion of R-5-P to hexosemonophosphate and triose phosphate by the nonoxidative reactions of the PPP in both primaquine-sensitive and normal erythrocytes was not changed by this procedure.

Table II. The net increase in pentose content of intact erythrocytes in the presence of methylene blue relative to that formed by erythrocytes without methylene blue; the accumulation represented an increase of 30 to 35 per cent

Subjects	*Pentose accumulation (μM/180 minutes/ml. of packed erythrocytes)*
Normal men	
No. 1	+ .89
No. 2	+ .74
No. 3	+ .69
No. 4	+ .68
Sensitive men	
No. 1	− .03
No. 2	− .04
No. 3	.00

Discussion

There is ample evidence to indicate that the deficiency of glucose-6-phosphate dehydrogenase in primaquine-sensitive erythrocytes results in defective activity of the PPP, i.e., increased ratio of TPN^+:TPNH,[6, 21] diminished CO_2 production,[22] and diminished oxygen utilization.[30] Since G-6-PD initiates the decarboxylation of G-6-P, which results in the production of equal quantities of CO_2 and pentose phosphate, it might be expected that the rate of pentose formation via this pathway would be diminished when G-6-PD is deficient. However, there was no decrease in the total pentose content of primaquine-sensitive erythrocytes. Furthermore, the over-all capacity to convert ribose-5-phosphate to triose phosphate and hexosemonophosphate by the reversible transketolase-transaldolase exchange reactions was equal in normal and sensitive erythrocytes.

Stimulation of the oxidative reactions of the PPP by methylene blue in normal erythrocytes was reflected by an increase in oxygen utilization and pentose accumulation. Under the same conditions, oxygen utilization was increased in primaquine-sensitive erythrocytes, but not to the same magnitude as in normal cells; in addition, pentose accumulation did not occur. Presumably, additional pentose was formed in primaquine-sensitive erythrocytes as a result of methylene blue stimulation, but the excess was small enough to be catabolized by the nonoxidative reactions of the PPP.

In the absence of methylene blue, the finding of equal concentrations of total pentose in primaquine-sensitive and normal erythrocytes does not indicate that the mechanism of pentose formation is the same in these 2 types of cells. In normal human erythrocytes, Hiatt and Lareau[23] demonstrated that the oxidative reactions of the PPP contribute slightly more ribose to nucleosides than do the nonoxidative reactions. While the oxidative G-6-PD and 6-phosphogluconic dehydrogenase reactions form pentose phosphate, these reactions do not catabolize or diminish the pentose phosphate pool, since they are physiologically irreversible. However, the nonoxidative portion of the PPP, composed primarily of the transketolase and transaldolase exchange reactions, is a reversible enzymatic pathway which can form as well as catabolize pentose. Although the activity of the reversible nonoxidative reactions of the PPP was equal in hemolysates from primaquine-sensitive and normal individuals, it is possible that these enzyme systems operate at different submaximal rates in vivo. If smaller amounts of pentose phosphate are produced by the G-6-PD and 6-phosphogluconic dehydrogenase reactions in primaquine-sensitive erythrocytes, the equilibrium of the nonoxidative reactions might be altered and result in a concentration of pentose similar to that in normal erythrocytes. Thus the contribution of these reversible reactions (TK and TA) to the pentose pool in circulating erythrocytes might be different in the 2 types of cells. Additional studies are needed in order to determine the proportional contribution of the oxidative reactions of the PPP, the nonoxidative transketolase and transaldolase reactions, and the degradation of nucleosides to the pentose phosphate pool. Primaquine-sensitive erythrocytes provide a useful model for further studies pertaining to the controlling mechanisms of the pentose phosphate pathway as well as pentose phosphate formation.

We are indebted to the late E. S. G. Barron, M.D., for some of the data presented in this paper.

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Ion exchange analysis of organic phosphate compounds of erythrocytes from individuals with neoplastic diseases

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A study using anion exchange analysis has been made of the various organic phosphate compounds in erythrocytes of patients with neoplastic diseases. Analyses were made after incubation of the erythrocytes for 3 hours at 37° C. 2, 3-Diphosphoglycerate, uridine diphosphate glucose plus uridine diphosphate N-acetylglucosamine, and an unknown component were significantly higher in the neoplastic group than in the normal group. The possible significance of these results is discussed.

Because of previous observations suggesting that exposure of the whole organism to ionizing radiation produced alterations in erythrocyte glycolysis,[1-3] a study of the effect of such exposure upon other aspects of erythrocyte metabolism was undertaken. Patients requiring total body irradiation for palliation of symptoms due to various types of malignant disease were selected. Although irradiation itself appeared to impose few alterations in either erythrocyte glycolysis or other parameters of erythrocyte metabolism, there were several significant differences between the data obtained in the study of erythrocytes of normal individuals and of those with lymphosarcomas and disseminated carcinomas.

Recent studies have shown the usefulness of ion exchange analysis in the isolation and quantitative determination of various organic phosphate compounds of erythrocytes from normal individuals.[4-7] In addition, this procedure

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Supported by United States Air Force Research Contract No. 41(657)-303, by United States Public Health Service Research Grant No. A-2419, and by a grant from the Brown-Lupton Foundation.

Received for publication Jan. 18, 1961.

has been utilized to demonstrate changes in organic phosphate compounds during in vitro incubation of blood at 37° C.[4] and during storage of blood at 4° C.[8-10] The present study describes the application of an ion exchange analysis to determination of organic phosphate compounds in erythrocytes of diseased individuals.

Rottino and co-workers[11] have studied total adenine nucleotides of erythrocytes of normal individuals and of patients with various neoplastic diseases. These investigators reported no significant differences in the amount of total erythrocyte adenine nucleotides when the values were expressed as amount per unit volume of erythrocytes. The present studies, using ion exchange analysis, show that several other organic phosphate components occur in above normal concentrations in erythrocytes of patients with certain neoplastic diseases.

Materials and methods

Subjects. Clinical data for patients with neoplastic diseases are summarized in Table I. The sex and age of normal individuals which were studied were as follows: L. T., female, 39; C. B., male, 12; D. B., female, 51; G. M., male, 36; H. R., male, 28.

Materials. Reagent grades of formic acid and ammonium formate were used in these studies. It is necessary to check ammonium formate solutions for phosphate content prior to their use in eluant solutions since some lots of ammonium formate contain enough inorganic phosphate to interfere with phosphate analyses on eluant fractions. Tris hydroxymethyl aminomethane (TRIS) was obtained from Sigma Chemical Company. The TRIS buffer-glucose solution was made up as follows: 10 ml. of 0.15 M TRIS buffer, pH 7.4; 2 ml. of 5.4 per cent glucose solution; and 18 ml. of 0.15 M NaCl.

Incubation procedure. Since many of the blood specimens used in this study contained markedly increased numbers of leukocytes, preliminary removal of the major portion of these was considered necessary. This was carried out utilizing a "separator" made from Lusteroid centrifuge tubes (Fig. 1). Heparinized blood was placed in the top portion of the assembled separator and centrifuged at 800 r.p.m. (relative centrifugal force = 95) for 15 minutes in an International refrigerated centrifuge, Model PR-2, at 4° C.[12] All plasma and cells remaining in the top portion of the separator were transferred with a fine-tipped pipette to another tube. The top section of the separator was removed, leaving the packed erythrocytes in the lower portion. The leukocytes and erythrocytes in the plasma were sedimented by high speed centrifugation and the supernatant plasma was carefully removed. Erythrocytes removed in the first separation were resuspended in this cell-free plasma and the separation process was repeated; this time, however, a slightly shorter lower portion was used for the separator. Use of centrifuged plasma for resuspension of erythrocytes provides a better separation of leukocytes from erythrocytes than that obtained by suspension of erythrocytes in isotonic saline. By utilizing this separation procedure, erythrocyte suspensions nearly free of leukocytes were obtained.

The erythrocytes (3 to 4 ml. of packed cells) were resuspended in 5 ml. of cell-free plasma from the same individual and 2 ml. of TRIS buffer-glucose solution. The added glucose was sufficient to raise the blood glucose by approximately 50 mg. per cent. For ion exchange studies, a 5 ml. portion of the erythrocyte suspension was incubated in a 25 ml. Erlenmeyer flask (A), under nitrogen, utilizing a Dubnoff metabolic incubator. The sample was shaken 60 times per minute at 37° C. The remaining portion of the cell suspension (flask B) was used for determination of the hematocrit and for glycolysis studies, as is described below. After 3 hours of incubation, the suspended cells in flask A were mixed with 10 ml. of cold 15 per cent trichloroacetic acid. After thorough mixing to ensure disruption of the cells, the sample was centrifuged at 2° C. in a refrigerated centrifuge. The supernatant fluid was removed and extracted 3 times with equal portions of cold diethyl ether. The ether extracts were discarded. If the pH of the aqueous phase was below 2.8, it was extracted a fourth time

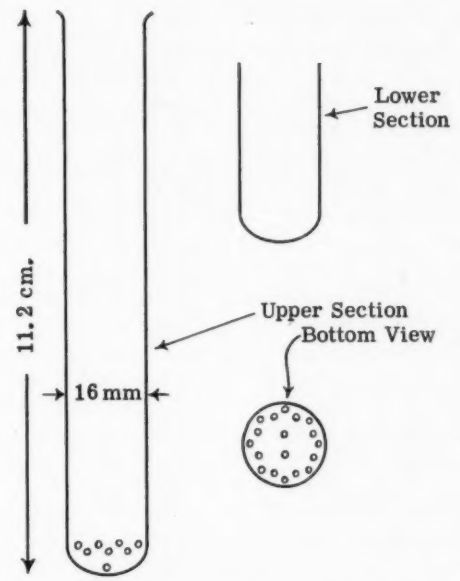

Fig. 1. Lusteroid "separator" for separation of leukocytes from erythrocytes. Both portions are made from 15 ml. Lusteroid centrifuge tubes (W. H. Curtin, No. 3926B). Since there is a slight variation in size of these tubes, the narrower ones are used for the upper portion. This simplifies assembling of the separator, since the upper section must be forced into the lower tightly enough to avoid leakage. The holes in the top section are punched with a short 20 gauge needle with care to keep them just below the line where the lower section fits onto the upper section. Different sizes of the lower section (2.0 to 3.5 cm. in length) are kept on hand so that the size which is utilized may be varied to match the expected volume of packed cells. It is important that the edge of this lower section be smooth, since a ragged edge promotes leaking and will soon tear. After use, these sections are separated very readily with little danger of spilling the contents of the lower section.

with ether. Excess ether was removed from the aqueous solution by subjecting it to a light vacuum (approximately 30 to 40 mm. Hg) for 5 to 10 minutes. The solution was then stored in a Deepfreeze until used for ion exchange analysis.

Glycolysis studies were performed simultaneously using the cell suspension in flask B. After removing samples for hematocrit determinations and two 0.5 ml. aliquots for initial glucose determinations, the remaining cell suspension in flask B was incubated in the same manner as flask A. After 3 hours of incubation, two 0.5 ml. aliquots were again removed from flask B for glucose determinations. In each instance, the aliquots of suspended cells were immediately deproteinized and glucose determinations on each filtrate were carried out in duplicate using the Nelson procedure,[13] with the alkaline copper reagent prepared as described by Somogyi.[14] Glycolysis rate was then calculated as micromoles of glucose utilized per milliliter of erythrocytes per hour.

Anion exchange analysis. The general principles of anion exchange analysis of erythrocyte organic phosphate compounds have been described previously.[4] In order to apply this procedure to the determination of erythrocyte organic phosphates in patients, the entire procedure was scaled down so that analyses could be made using a blood filtrate prepared from 1.5 to 2 ml. of packed cells. Gradient elution was utilized with a Dowex 1-X4 column, 0.5 cm. in diameter and 20 cm. long.* Water (75 ml.) was present initially in the mixing reservoir

*In order to assure a satisfactory flow rate, this column should be prepared fresh each time prior to use. All washes of the ion exchange resin should be carried out prior to pouring the column.

Table I. Clinical data of patients

Patient	*Sex*	*Age in years*	*Diagnosis*	*Hemoglobin (Gm. %)*	*MCV (μ^3)*	*MCH ($\mu\mu g$)*	*MCHC (%)*	*Total W.B.C. cells/ $mm.^3$*	*% of reticulocytes*	*Glycolytic rate*
M. Z.	F	47	Malignant lymphoma, disseminated	5.7	131	34	25	5,850	0.8	3.57
A. A.	M	58	Malignant lymphoma, disseminated	9.7	98	28	29	21,300	0.2	2.01
J. H.	M	49	Malignant lymphoma (reticulum cell sarcoma of bladder)	13.9	100	31	30	14,500	0.4	2.26
A. J.	M	62	Malignant lymphoma, disseminated	8.2	86	26	31	4,950	1.2	3.29
A. P.	M	76	Hodgkin's granuloma	13.9	109	34	31	9,350	0.6	2.03
S. C.	M	82	Lymphosarcoma	12.9	94	27	29	5,800	2.0	2.73
O. I.	F	62	Hodgkin's lymphoma	12.2	100	34	34	17,150	2.4	2.04
R. H.	F	53	Adenocarcinoma, disseminated	10.6	85	27	31	4,250	0.8	1.81
C. J.	F	45	Papillary cystadenocarcinoma with omental metastases	11.2	95	30	32	7,450	1.8	2.19
T. W.	M	14	Epidermoid carcinoma of nasopharynx with metastases	10.1	92	28	29	9,750	1.4	2.81

All values listed are pre-irradiation values. Glycolytic rate is expressed as micromoles of glucose utilized per milliliter of erythrocytes per hour. MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

and the solutions which were utilized in the upper reservoir were as follows: (1) 60 ml. of 0.5 N formic acid; (2) 150 ml. of 4 N formic acid; (3) 150 ml. of 4 N formic acid + 0.8 M ammonium formate. Fractions of 3 to 4 ml. were collected with an automatic fraction collector. Sufficient pressure was used above the column so that changes in the contents of the upper reservoir could be made at 8 and 24 hours after the start of elution. Generally, a hydrostatic pressure above the column of about 16 inches was adequate for this purpose. The optical density of the eluant fractions was determined routinely at 260 mμ using a Beckman DU spectrophotometer. In addition, readings at 275 and 290 mμ were made on the contents of those tubes containing appreciable 260 mμ–absorbing material, in order to facilitate proper identification of compounds. Volumes of particular samples containing each

component were determined from the weight and specific gravity of the fraction. Once the specific gravity has been determined for a given peak, that value may then be used in subsequent calculations of fraction volumes.

Since formic acid solutions absorb some light at 260 mμ, it was necessary to correct all optical density readings for the absorbance due to formic acid which was present in each sample. This was done, whenever possible, by taking the average absorbance of blank tubes both prior to and following a given peak. However, in a few cases where components were eluted close together, a blank absorbance value was available on only one side of a peak.

Millimolar extinction coefficients (E_{mM}) at 260 mμ which have been used in these studies are as follows: adenosine 5′-mono-, di-, and triphosphates (AMP, ADP, and ATP), 14.4; di- and triphosphopyridine nucleotides (DPN and TPN), 18.3; inosine 5′-monophosphate (IMP), 8.1; guanosine 5′-triphosphate (GTP), 11.7; and uridine nucleotides (UDPX), 9.9.[4, 15] Cytidine and deoxycytidine nucleotides (CDPX) were determined from D_{275} readings (optical density readings at 275 mμ) using an E_{mM} of 12.3. Since component B is eluted from the column at the same time as ATP, the values for ATP have been corrected by subtracting the amount of component B from the total for this peak of the elution profile. Since uric acid appears in the elution profile in the same position as DPN, values for the latter compound were corrected for uric acid content in the manner described previously.[4] No extinction coefficients are known for components A and B. Consequently, it has been assumed for purposes of comparison that these components have millimolar extinction coefficients at 260 mμ equal to that of adenine nucleotides (14.4). Component B, which appears in the latter portion of the ATP peak, has a D_{290}:D_{260} ratio of 0.27. The D_{290} readings and a value of 3.9 for the E_{mM} at 290 mμ (0.27 × 14.4) have been utilized to calculate the amount of this compound. After correction of observed D_{290} and D_{260} readings for formic acid blank absorbance, the optical density at 290 mμ due to component B, (x), is calculated as follows: $x = D_{290} - 0.030\ D_{260}$. This equation is derived using the known D_{290}:D_{260} ratio for ATP (0.030) and assuming that the optical density at 260 mμ is due to ATP only. Since component B is present in erythrocytes in small amounts compared to ATP, no appreciable error is introduced by making this assumption.

2, 3-Diphosphoglycerate (2, 3-DPG) phosphorus was determined in eluant fractions on 0.5 ml. aliquots using the total phosphate procedure of Fiske and SubbaRow[16] modified to give a final volume of 10 ml. of colored solution. Hexose diphosphate (HDP) phosphorus was determined on 1.5 ml. aliquots by the total phosphate procedure of Horecker and associates[17] modified slightly to give a sulfuric acid concentration of 1.5 N during color development.[18] If ATP was present in samples which were to be analyzed for HDP, the ATP was removed by adsorption on Norit*-activated carbon (10 mg. of Norit per milliliter of sample) prior to analysis. If any ATP remained in the filtrate, the process was repeated. The Norit A had been tested previously to show that it did not contribute phospate to the sample or remove HDP from the sample under these conditions.

Results and discussion

The incubation procedure described may be considered to be a metabolic challenge to erythrocytes. Erythrocytes from normal individuals reflect certain changes when incubated under these conditions. Incubation in plasma-TRIS buffer–glucose medium appears to be somewhat less physiologic than incubation in plasma with added glucose.[4] The data in Table II summarize the changes observed during the 3 hour incubation period with 2 samples of normal blood. The primary changes are: (1) a fall in amount of ATP followed by an increase in amount of AMP; (2) a slight increase (usually) in amount of IMP; (3) a

*Norit A (acid washed), Pfanstiehl Labs, Inc., Waukegan, Ill.

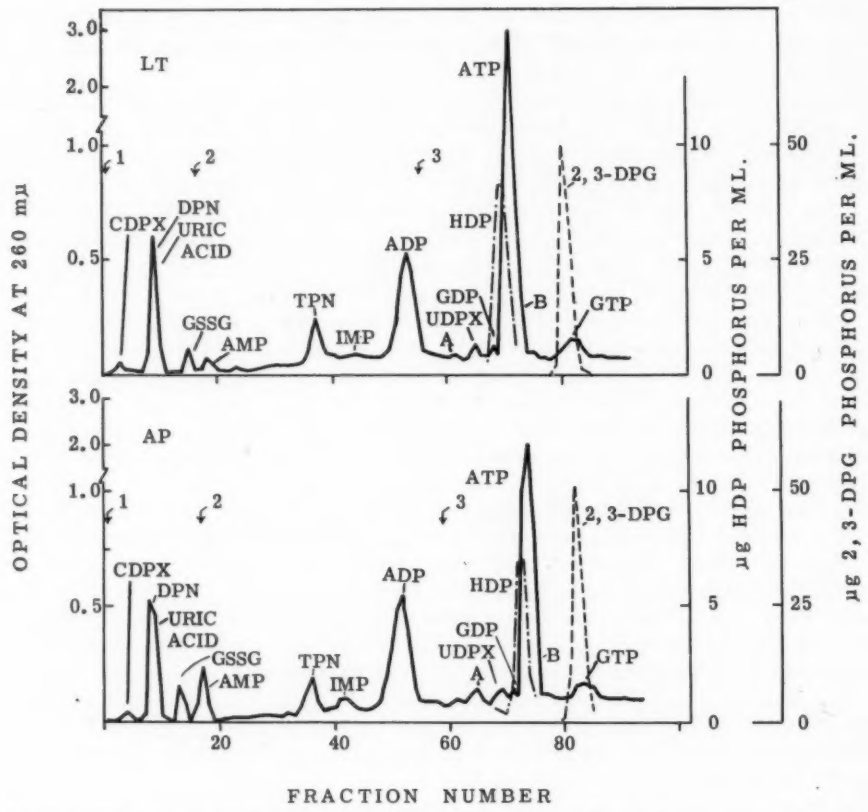

Fig. 2. Ion exchange separation of erythrocyte organic phosphates from a normal individual (L. T.) and from an individual with lymphoma (A. P.). The solution in the upper reservoir was changed at the numbered arrows as follows: *1*, 0.5 N formic acid; *2*, 4 N formic acid; *3*, 4 N formic acid + 0.8 M ammonium formate. In addition to abbreviations listed previously, *GSSG* is used to denote oxidized glutathione. The solid line in the figure represents optical density of the eluant solution at 260 mμ; the dashed line represents 2, 3-DPG phosphorus; and the dot-dash line represents HDP phosphorus.

marked increase in hexose diphosphate* concentration; and (4) a decrease in the inorganic phosphate concentration of the cell suspension. At present the cause of these changes is not known. A basic hypothesis of this study was that such a challenge to erythrocytes would make overt abnormalities in erythrocyte metabolism not apparent otherwise. Whether this incubation procedure is the optimum method for challenging erythrocytes is, however, still uncertain. The data presented in subsequent portions of this study represent determinations made after the 3 hour incubation period, and are not necessarily values for the various organic phosphate compounds in erythrocytes under in vivo conditions.

The data in Table II also show that only minor changes in most components occur during procedures used for removal of leukocytes. The increase in the

*Studies using analytic methods specific for fructose indicate that most of this increase may be accounted for by an increase in fructose 1, 6-diphosphate.

amount of component B is very striking, however. At present we have no explanation for this phenomenon. The slight apparent fall in the ATP level may be related to removal of leukocytes and some of the younger erythrocytes during the washing procedure, or it may be due to the changes in environment (temperature, CO_2 tension, etc.) brought about by the application of these procedures.

The results of anion exchange analyses of erythrocyte organic phosphates in patients with neoplastic diseases are summarized in Table III. Anion exchange profiles which were obtained with erythrocytes from a malignant lymphoma patient (A. P.) and from a normal individual (L. T.) are depicted in Fig. 2. From 2 to 5 anion exchange analyses were performed prior to and following irradiation of the lymphoma and carcinoma patients. In most instances, successive elution profiles for a given patient were similar; pre-irradiation values of components in the profiles are presented in Table III. In 2 instances, the clinical condition of patients changed markedly during the course of the studies. The first, A. J., was critically ill when the pre-irradiation analysis was carried out. This profile was characterized by high AMP, IMP, HDP, and 2, 3-DPG peaks and a low ATP peak. Following radiation therapy, the patient's clinical condition improved markedly. At 10 and 14 weeks postirradiation, the amount of ATP had increased markedly, and the amounts of the other components had decreased (Table III). The second patient (M. Z.) was in relatively poor condition initially when the first ion exchange study was carried out. The last ion exchange analysis was carred out 11 days after the initial one and 2 days prior to death. The patient was critically ill; this may be reflected by low ATP and high ADP, AMP, and IMP values which were observed at this time.

Table II. Changes in erythrocyte phosphate compounds of normal blood during the washing procedure and during the subsequent incubation at 37° C.

	Subject	*G.M.*		*Subject*	*D.B.*	
Component	*Initial**	*Pre-incubation†*	*Post-incubation*	*Initial**	*Pre-incubation†*	*Post-incubation*
CDPX	2.2	2.1	1.4	2.0	1.8	1.4
DPN	7.4	9.1	7.1	6.7	7.1	5.5
AMP	1.0	1.2	9.7	0.8	3.8	8.8
TPN	3.7	4.0	3.5	4.3	4.4	3.8
IMP	0	1.6	3.8	1.3	1.8	4.1
ADP	16.1	17.5	30.5	10.6	18.5	18.2
ATP	144.	116.	76.5	107.	84.7	30.6
HDP	26.6	28.5	94.	31.4	40.4	117.
2, 3-DPG	426.	440.	459.	575.	551.	532.
A‡	1.1	0.6	0.4	0.9	1.1	1.2
B‡	4.2	12.4	6.9	6.2	10.9	5.8
Inorganic P	92.7	49.7	37.3	110.	71.1	52.7

*Whole blood.

†After resuspending the washed erythrocytes in plasma and TRIS buffer—glucose.

‡Assuming an E_{mM} at 260 mμ of 14.4

The values are expressed in terms of micromoles of compound per 100 ml. of erythrocytes, with the exception of inorganic P which is expressed as micromoles per 100 ml. of cell suspension.

Table III. Organic phosphate compounds in erythrocytes of normal and diseased individuals

Subjects	Component*												
	AMP	*ADP*	*ATP*	*IMP*	*HDP*	*2, 3-DPG*	*CDPX*	*DPN*	*TPN*	*UDPX*	*A*	*B*	*GTP*†
Lymphoma													
M. Z.	9.0	22.0	60.0	4.3	181	768	2.4	6.8	4.1	5.0	1.6	8.5	9.1
M. Z.‡	19.0	34.5	39.1	11.7	186	712	1.6	5.2	3.7	3.7	1.5	11.6	8.8
A. A.	11.6	28.7	36.9	11.0	115	570	0.9	6.2	3.0	2.8	1.2	8.0	9.1
J. H.	10.6	27.6	53.9	6.8	99	594	1.7	5.6	3.8	5.7	0.8	10.7	5.7
A. J.	9.2	24.2	23.3	7.0	278	819	—	12.3	4.3	—	1.6	7.5	7.9
A. J.§	1.8	13.6	82.5	1.7	59	600	2.3	7.4	3.8	4.5	0.5	11.8	9.6
A. P.	6.8	26.9	69.2	4.2	78	558	2.2	7.1	4.0	3.6	2.3	12.0	8.2
O. I.	5.3	96.0	93.3	2.9	47	484	3.4	7.9	4.5	2.5	1.6	5.2	10.5
S. C.	9.4	35.1	39.0	8.8	133	541	2.7	7.6	4.3	4.8	1.2	2.8	7.3
Carcinoma													
T. W.	1.2	15.2	118	1.0	54	550	2.2	6.5	3.1	6.5	2.1	12.9	8.4
R. H.	4.1	20.9	102	4.9	87	803	1.2	6.1	3.6	4.9	1.3	13.0	9.9
C. J.	7.5	29.3	78.0	3.0	132	684	2.2	7.1	3.9	4.5	2.1	11.0	7.9
Average‖	7.5	32.6	67.4	5.4	120	637	2.1	7.3	3.9	4.5	1.6	9.2	8.4
Normal subjects													
L. T.	1.3	22.7	104	1.0	95	515	1.9	6.9	5.7	2.0	0.8	10.8	8.9
C. B.	2.8	37.0	85.8	0	61	555	1.8	6.9	4.1	2.3	0.8	12.2	8.8
D. B.#	5.3	23.2	47.3	2.1	100	559	1.9	6.5	4.1	3.1	0.8	8.9	7.1
G. M.#	9.6	31.5	80.0	3.8	89	463	1.7	6.6	3.6	3.8	0.7	8.5	5.7
H. R.	14.5	34.8	70.1	4.8	167	270	3.9	8.3	3.9	3.6	1.0	8.9	—
Average	6.7	29.8	77.4	2.3	102	472	2.2	7.0	4.3	3.0	0.8	9.9	7.6

Abbreviations: AMP, ADP, and ATP = adenosine 5′-mono-, di-, and triphosphates, respectively; IMP = inosine 5′-monophosphate; HDP = hexose diphosphate; 2, 3-DPG = 2, 3-diphosphoglycerate; CDPX = cytidine and deoxycytidine nucleotides; DPN and TPN = di- and triphosphopyridine nucleotides, respectively; UDPX = uridine nucleotides; A and B refer to components; and GTP = guanosine 5′-triphosphate.

*All values are expressed as micromoles per 100 ml. of erythrocytes.

†Includes an unidentified component in addition to GTP.

‡Eleven days after initial studies; patient in very poor condition.

§Average of 2 analyses 10 to 14 weeks after initial studies; patient's clinical picture was markedly improved.

‖Average of pre-irradiation values for the neoplastic group.

#Average of 2 analyses.

Several additional observations in these studies are worth noting. First, a new component (peak *A*, Fig. 2, Patient A. P.) was detected in erythrocytes of a number of these patients. This compound appears in elution profiles just ahead of the UDPX peak. It has a D_{290}:D_{260} ratio between 0.25 and 0.50, which is higher than this ratio for adenine or uracil nucleotides. This component could contain guanine, or it might be related to the nucleotide found in the latter portion of the ATP peak (peak *B*), which is also characterized by a high D_{290}:D_{260} ratio.[4, 19] Component A is not guanosine diphosphate (GDP), since GDP appears just ahead of ATP in elution profiles (Fig. 2). In the present studies, the amount of material in peak *A* has been too small to determine ribose

or phosphorus or to obtain a good ultraviolet absorption curve. Profiles from normal individuals often showed a very small peak for this component. Since this component is more prominent in profiles obtained with material from patients with lymphomas and carcinomas, additional studies must be carried out to determine its chemical nature.

The unknown nucleotide in the latter portion of the ATP peak (peak *B*) was apparent in the profiles of every one of the individuals which were studied (Table III). Component B has a higher absorption at 290 mμ than adenine nucleotides, and is readily apparent as a distinct peak in elution profiles when optical densities at 290 mμ are plotted. The UDPX peak recently has been shown to contain uridine diphosphate glucose and uridine diphosphate N-acetylglucosamine.[20] This peak was present in all elution profiles (Table III). Recent studies have shown that in other tissues, these uridine nucleotides are concerned with the synthesis of complex lipids, glycoproteins, and polysaccharides.[21] Consequently, they may play an important role in the formation and maintenance of the erythrocyte stroma and may also be concerned with some of the immunologic characteristics of erythrocytes.[22] The cytidine and deoxycytidine compounds (CDPX) have been described previously[23] and probably are concerned with phospholipid synthesis.[24, 25] DPN and TPN are important coenzymes and are involved in the glycolytic and phosphogluconate pathways of metabolism, respectively. TPN also plays a key role in the protection of erythrocytes from the effects of various drugs.[26, 27] Recent studies have shown that guanine is readily incorporated into erythrocyte GTP in vitro.[28, 29] Consequently, GTP may be presumed to play an active role in erythrocyte metabolism.

A comparison of organic phosphate compounds of individuals has been made by application of statistical analysis to the data shown in Table III. These results are summarized in Table IV. 2, 3-DPG, UDPX, and component A showed significantly higher values for the neoplastic than for the normal group. Although the values for IMP were appreciably higher for the neoplastic than for the normal group, the difference is of questionable significance ($p = 0.06$). In addition, the amount of component B was increased in the group of carcinoma

Table IV. Summary of differences in erythrocyte organic phosphate compounds

Groups compared	*No. of individuals*	*Concentration of component* (Average ± S.D.)†*	*Component*	*p‡*
Normal	5	472 ± 119	2, 3-DPG	0.03
Neoplastic	10	637 ± 122		
Normal	5	2.3 ± 2.0	IMP	0.06
Neoplastic	10	5.4 ± 3.0		
Normal	5	3.0 ± 0.8	UDPX	0.04
Neoplastic	9	5.4 ± 1.3		
Normal	5	0.82 ± 0.11	A	0.01
Neoplastic	10	1.58 ± 0.48		

*Micromoles per 100 ml. of cells.
†S. D. = standard deviation.
‡Probability calculated by applying student's "*t*" test.

patients as compared to the normal group. However, the number of carcinoma patients which were studied was too small to assure the statistical significance of this difference. Differences between normal and neoplastic groups in average values for other components shown in Table III were not statistically significant ($p > 0.10$) for the number of individuals which were studied.

Although statistical analysis suggests that these differences are related to the presence of neoplasm in these patients, definite conclusions are not possible. At the present time similar studies dealing with effects on erythrocyte metabolism of other factors, such as age of the individual, intake of various medications, and metabolic state of the patient have not been performed. Until such data are available and until the effects of other diseases have been determined, the role of neoplasms in the production of these changes is uncertain.

We wish to acknowledge the technical assistance of Mrs. Carolyn Battle in carrying out many of the above experimental procedures.

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Red cell glutathione content and stability in renal insufficiency

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The reduced glutathione content and the stability of glutathione to acetylphenylhydrazine were determined in 16 consecutive anemic patients with either acute renal insufficiency or chronic renal decompensation. In the majority of the patients, the glucose-6-phosphate dehydrogenase activity of the erythrocytes was simultaneously evaluated. A significant decrease of glutathione and its stability to acetylphenylhydrazine in those with acute uremia and a significant lowering of glutathione stability in the group with chronic renal disease were found when compared with the values obtained in 15 normal subjects. The glucose-6-phosphate dehydrogenase activity was significantly greater, however, than in the normal subjects, reflecting a younger mean cell age.

This observation emphasizes that a correlation need not exist between the glutathione content or its stability and this enzyme, since one may find high enzyme values with normal or decreased glutathione and glutathione stability.

Few studies have been accomplished at the tissue level which define specific metabolic derangements in uremia.[1, 2] Since anemia invariably occurs in renal failure, the readily accessible erythrocyte has provided an approach to one parameter of an involved system.[3-9] A decrease in red cell survival seems to be a common lesion in this population group.[10] Among the cellular abnormalities noted are alterations in phosphate and cationic partitioning,[2] glucose uptake,[2] and, in the bilaterally nephrectomized dog, a significant fall in the amount of reduced glutathione (GSH).[11] Because of the protective role ascribed

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Supported in part by Contract Nonr-222(51) between the Office of the Naval Research and the University of California at San Francisco.
The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or as necessarily reflecting the views of the Medical Department of the Navy or of the Naval Service at large.
Received for publication Dec. 12, 1960.

to GSH in the maintenance of red cell integrity in inherited glucose-6-phosphate dehydrogenase (G-6-PD) deficiency disease[12] as well as in other studies,[13-15] an evaluation of this intracellular sulfhydryl tripeptide was carried out in anemic, uremic patients to determine whether there was a significant difference from the normal.

Materials and methods

Patients. On admission, and prior to treatment, heparinized venous blood was drawn into test tubes containing sufficient glucose to yield a final concentration of about 200 mg. per cent from 16 consecutive patients with acute or chronic renal insufficiency who manifested variable degrees of anemia and impairment of renal function. Patients Nos. 1, 3, 4, and 6 received transfusions 1 week prior to the study, due to the circumstances in which the acute renal failure occurred. Only 1 Negro (Patient No. 10, a woman) participated in this study. In each case, the plasma urea nitrogen (PUN) was above 25 mg. per cent and the micro-hematocrit value was below 37 per cent, with normal cell indices. Of the 16 patients, 7 had acute renal failure and 9 had chronic decompensated renal disease. The diagnoses are listed in Table I. All specimens were drawn approximately 1 to 2 hours after breakfast, with the exception of those from the patients with acute uremia who, on the usual regimen of nothing per os, received a slow intravenous drip of 20 per cent dextrose in water running continuously. The blood glucose values under these conditions were either normal or moderately elevated.

Control subjects. Similar blood samples were obtained from 15 healthy control subjects without anemia or renal disease. Throughout the study, a blood sample from a normal control subject was analyzed in duplicate, together with the reduced glutathione standards. The analyses were begun within 3 hours from the time the blood was drawn in both the patients and the normal subjects. During this interval the specimen tubes were kept refrigerated at 4° C.

The GSH content was determined by the Beutler modification[12] of the Grunert and Phillips technique.[16] The GSH stability to acetylphenylhydrazine (APH), as described by Beutler,[12] was carried out employing 5 mg. of APH per tube. Ergothioneine, a sulfhydryl derivative of histidine present in the erythrocyte in significant concentrations, did not develop color when prepared in the manner of the GSH standards, nor could it be recovered by this method when it was added to the whole blood sample prior to making the metaphosphoric acid filtrate. G-6-PD activity was measured in the Beckman DU spectrophotometer at 340 mμ by Marks' adaptation[17] of the Kornberg and Horecker method.[18] The blood samples were placed immediately in chilled test tubes, without the additional glucose, and spun down in a refrigerated centrifuge. Ten microliters of hemolysate from an adjusted hematocrit level in the vicinity of 20 per cent was employed for the enzyme assay. The final cuvette concentration of the substrate disodium glucose-6-phosphate was 2×10^{-3} M. The units are expressed as the change of O. D. per minute per milliliter of red cells. Urea in final cuvette concentrations of 200 to 300 mg. per cent did not alter the activity of this enzyme.

Results

The control levels of GSH and GSH stability, as well as the per cent intact (a derived figure), coincide with other observations made on normal population groups. Distribution analyses of the content and stability in the uremic patients revealed curves of similar base width (range) which, however, were significantly lower than in the normal subjects, as ascertained by the student "t" test for nonpaired experiments[19] (Table I). The exception was the GSH content of the group with chronic disease which did not differ from that of the control subjects. Multiple determinations in the control group over a period of time yielded virtually the same means and standard deviations as the figures obtained when

Table I. Erythrocyte reduced glutathione content, stability, and per cent of the original content intact in patients with acute and chronic renal insufficiency

Patient No.	*Diagnosis*	*GSH content* (mg./100 ml. red cells)	*Post-incubation GSH content* (mg./100 ml. red cells)	*Original GSH intact* (%)	*PUN* (mg./100 ml.)	*Hematocrit* (%)	*G-6-PD activity* (units)
Patients with acute renal insufficiency							
1	Postaortic resection	39	11	28	238	28.0	
2	Carbon tetrachloride nephrosis	61	37	61	206	34.5	
3	Postoperative nephrectomy	54	36	67	117	30.0	
4	Postaortic resection	63	28	44	203	35.0	4.67
5	Postfebrile illness and dehydration	35	25	71	120	25.0	4.44
6	Hemorrhagic shock, incompatible blood transfusion	54	44	81	189	35.0	5.77
7	Glomerulonephritis with acute renal shutdown	63	34	54	112	25.0	8.14
Patients with chronic renal insufficiency							
8	Obstructive uropathy	96	20	21	212	19.1	5.85
9	Obstructive uropathy	64	54	84	34	32.5	4.07
10	Chronic pyelonephritis	104	55	53	166	26.5	4.21
11	Nephrosclerosis, chronic pyelonephritis	57	19	33	82	33.0	3.85
12	Nephrosclerosis; chronic pyelonephritis	53	43	81	100	28.0	
13	Malignant nephrosclerosis	71	60	85	30	36.0	4.23
14	Chronic glomerulonephritis	83	60	72	143	28.0	6.10
15	Nephrosclerosis; chronic pyelonephritis	88	54	61	27	31.0	5.58
16	Nephrolithiasis; chronic pyelonephritis; nephrosclerosis	76	42	59	74	33.5	4.60
Normal subjects (15)							
	Mean	75	66	89		44.2	3.66
	Range	58-90	50-90	57-106			3.11-4.26
	Standard deviation	± 11	± 14	± 13			± 0.31
Patients with acute renal insufficiency (7)							
	Mean	53	31	58	169	30.4	5.76
	Range	35-63	11-44	28-81			4.44-8.14
	Standard deviation	± 11	± 11	± 18			± 1.69
	p value	< 0.001	< 0.001	< 0.001			< 0.05
Patients with chronic renal insufficiency (9)							
	Mean	77	45	61	96	29.7	4.81
	Range	53-104	19-60	21-85			3.85-6.10
	Standard deviation	± 17	± 16	± 23			± 0.89
	p value	(not significant)	< 0.01	< 0.01			< 0.05

they were used for the controls. For 2 reasons, this technique could not be applied to the patients under study. First, repetitious low determinations would bias the data, and second, many variables were introduced after the initial study, including blood transfusions, dialyses, and improvement of the uremic state. Thus far no consistent correlation has been established between these factors and changes in the GSH studies, except to note the return to normal in reversible renal insufficiency during diuresis and recovery. Table II is an example of observations in a case of prolonged oliguria associated with an intense catabolic response following aortic resection. Both the content and stability returned to normal on 2 separate occasions within 72 hours after hemodialysis. A similar change in content was noted in another individual (Patient No. 5) following 36 two hour intermittent peritoneal lavages, but significant instability was still present with an abnormal per cent intact. The frequently elevated white blood cell count during oliguria would tend to bias the data in a positive manner because of the higher GSH content of the white cells. The per cent intact, also significantly lower in both groups of patients, had the largest standard deviation.

In acute renal insufficiency, the mean values with reference to GSH, GSH stability, and per cent intact were 29, 53, and 35 per cent lower, respectively, than the corresponding values in normal subjects. The mean differences after incubation with APH and the per cent intact were lower by 32 and 31 per cent, respectively, in the patients with chronic uremia. The patients, therefore, were most similar with regard to the per cent intact and, as noted previously, most different in respect to the original content. In both groups the following several combinations of results are apparent: A normal or low content with instability and either normal or low per cent intact in the patients with acute uremia, and decreased APH stability together with a decreased per cent intact in those with chronic renal failure. In the course of these observations, 3 individuals with chronic renal disease (Patients Nos. 10, 15, and 16)—one with a high initial GSH value (Patient No. 10)—had an abnormal per cent intact despite normal levels after incubation with APH. The significance of the observation is not apparent and the per cent intact is admittedly borderline in each instance. No correlation was obtained in either group between the hematocrit or the PUN and any of the determinations. Table III represents observations on a patient with chronic glomerulonephritis initially evaluated while a "good chronic" without anemia, who then rapidly deteriorated with respect to renal function and in whom the GSH stability and the per cent intact seemed to decline progressively and significantly. Although it is apparent from the mean values shown in Table I, it should be emphasized at this point that the magnitude of the changes noted are of a lesser degree than those found in the hereditary drug-sensitive hemolytic anemias.

None of the GSH evaluations carried out in other nonanemic patients with significant renal disease or in patients with anemias of diverse etiologies but without renal disease has thus far given abnormal results. The G-6-PD activity of the erythrocytes was greater ($p < .05$) than that in the normal subjects (Table I).

Table II

Patient No.	Day of oliguria	GSH content (mg./100 ml. of red cells)	Post-incubation GSH concentration (mg./100 ml. of red cells)	Original GSH intact (%)	PUN (mg./100 ml.)	Hematocrit (%)	Remarks
1	16	39	11	29	238	28.0	Immediately pre-hemodialysis
	19	58	55	95	159	30.5	
	23	15	0	0	287	16.5	Immediately pre-hemodialysis
	27*	80	47	59	245	19.5	

*Urine volume exceeded 1,000 ml. per day for the first time on day 26.

Table III

Patient No.	Day following initial observation*	GSH content (mg./100 ml. of red cells)	Post-incubation GSH concentration (mg./100 ml. of red cells)	Original GSH intact (%)	PUN (mg./100 ml.)	Hematocrit (%)	G-6-PD activity (units)
14	0	69	71	103	92	42.5	
	33	96	90	94	98	41.0	
	109	83	60	72	143	28.0	6.10
	124	78	38	49	202	21.0	6.08

*Extensive Fe^{59} kinetic studies, through the courtesy of Dr. Myron Pollycove, Donner Radiation Laboratory, Berkeley, Calif., on day 60 (hematocrit, 37.5 per cent) revealed an erythron life span of 25 days (normal, 100 to 135) with a calculated rate of erythropoiesis approaching double the normal rate.

Discussion

The significantly altered GSH stability in both groups and the decreased initial amount of GSH in patients with acute tubular necrosis extend the experience of metabolic changes which may occur in renal failure. The abnormalities described were more severe and consistent in acute renal insufficiency and suggest that either the rate of progression of the uremic state or the degree of excretory suppression and renal compromise may be the determinants for this difference, since no correlation was found in either patient group with respect to the height of the plasma urea nitrogen or the degree of anemia. The less complete demonstration of GSH changes in the patients with chronic uremia, however, could reflect a more rapid disappearance of red cells with diminished GSH content and stability, thus preventing them from contributing significantly to the over-all population. One would expect the GSH stability test to be more significant in such a situation, and, indeed, this seems to be the case (Table I).

The significantly higher G-6-PD activity in both patient groups emphasizes that a correlation need not exist between the GSH content or its stability and this enzyme; one may find high enzyme values with normal or decreased GSH and GSH stability. The increased G-6-PD activity reflects the younger mean age[17] of the circulating erythrocyte similar to the index utilizing true acetylcholinesterase[20]: in hematologic diseases. That the activity of G-6-PD is elevated attests to the effort of the bone marrow and is more significant than the reticulocyte count in this respect.[17]

The normal intracellular level of GSH is a function both of adequate tripeptide synthesis and enzymatic reduction to maintain glutathione in the SH state. Diminished stability of GSH to APH would be evidence against a lessened synthesis of glutathione if the initial content were normal. This relationship obtained in 6 of the 16 patients evaluated, 4 in the group with acute renal failure and 2 with chronic kidney disease where the GSH stability test was abnormal (−2 S. D.) despite normal initial levels. The turnover rate of reduced glutathione, however, may be the important consideration relative to these observations. Although phosphorolysis (with reference to inosine) and the glycolytic pathway are apparently intact[2] in uremic red cells, a decrease in glucose uptake[2] has been reported in the uremic subject with decompensated metabolic acidosis and in the bilaterally nephrectomized animal.[11] This decrease may potentially affect the glycolytic as well as the hexose monophosphate shunt in terms of actual metabolic requirements. Parenteral inosine[2] and artificial hemodialysis[2] have been effective in reversing intracorpuscular abnormalities and improving erythrocyte survival in patients with chronic uremia. Metabolically, the former approach is thought to provide ribose phosphate substrate for cellular energy, but no studies are reported which elucidate the mechanism of action in the latter situation.

Adequate and stable GSH are believed necessary for the functional stability of the red cell, and instances in which lowering occurs have been considered prehemolytic states.[12-15, 21] The experimental work of Fegler[13] indicates that gross hemolysis begins when the GSH falls to 40 per cent of its original level. In absolute terms, this derived figure does not consider initial levels which may be toward the low side or abnormally low. However, the work of Buetler[22] and our own normal statistics indicate that the low normal value for GSH stability is in the environs of 40 mg. per cent. The GSH abnormalities demonstrated in our anemic, uremic patients also may be related to the integrity of the circulating erythrocyte.

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The survival of virus-treated erythrocytes in normal and splenectomized rabbits

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The survival of virus-treated erythrocytes in normal and splenectomized rabbits was studied using radiochromium-tagged cells. Erythrocytes were tagged with radiochromium using in vitro and in vivo techniques. The survival of normal erythrocytes in normal animals was the same by both techniques.

Normal erythrocytes in normal rabbits showed a mean apparent half time survival of 12 days. Those studies carried to completion showed a survival time of between 55 and 60 days. Normal erythrocytes in splenectomized animals showed a half time survival at the upper limits of normal.

Erythrocytes treated with Newcastle disease and influenza virus showed a shortened survival time in normal rabbits. Newcastle disease virus apparently produced more damage to the red blood cells than did influenza virus, as judged by the markedly shortened survival of cells treated with the former agent.

Influenza virus–treated erythrocytes appeared to survive longer in rabbits previously splenectomized. These findings suggest that the removal of this source of reticuloendothelial tissue prevented a more rapid removal of the virus-modified cells from the general circulation.

Recent studies have contributed much toward establishing the sites of red blood cell destruction in acute and subacute hemolytic anemias. The use of radiochromium for following the survival of erythrocytes and scanning of the body surfaces for radioactivity as an indicator of red blood cell terminus has shown that the roles of the liver and spleen, although overlapping, are relatively distinct. In an acute, supposedly intravascular destruction, the liver apparently handles most of the liberated pigments. However, in less acute situations the

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Supported in part by Grants Nos. H-2669 (C-3) and E-1559 (C-2) from the National Institutes of Health, United States Public Health Service.

Received for publication Feb. 15, 1961.

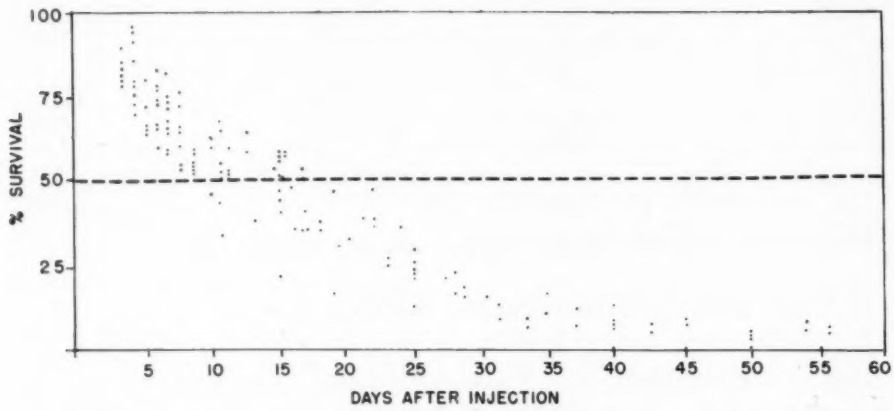

Fig. 1. The survival of autologous erythrocytes tagged in vitro with radiochromium in 17 normal rabbits. The apparent half time of survival ranged between 8 and 17 days, with a mean of 12 days.

red blood cells may be sequestrated in the splenic sinusoids and pulp for longer periods of time. This produces or enhances changes in the red cell, as emphasized by Jandl and associates,[1] which result in the spleen becoming the primary organ of destruction. In these cases splenectomy frequently has been of benefit.

There is, however, a large hiatus in this picture of splenic destruction. How is the red blood cell destroyed in the spleen? Is it primarily intravascularly via hemolysis and/or fragmentation, with the macrophages playing only a role of handling the pigment, or is it extravascularly, i.e., are the damaged cells more easily removed by the macrophages? Perhaps both mechanisms play some part; however, there is much evidence favoring importance of the latter, i.e., extravascular destruction.

Previous reports from this laboratory have emphasized enhanced susceptibility of the modified cell to phagocytosis by rabbit splenic macrophages in tissue culture.[2] The degree of phagocytosis of red blood cells modified by a variety of agents, i.e., enzymes, viruses, chemicals, and antibodies, varies between 20 and 90 per cent, depending on the type of treatment imposed. On the other hand, normal human and rabbit erythrocytes are phagocytized minimally, i.e., less than 12 per cent.

Since it was postulated previously that phagocytosis plays an important role in the removal of damaged or modified erythrocytes from the peripheral circulation, the susceptibility to phagocytosis of erythrocytes from patients with a variety of hematologic, infectious, and malignant diseases was determined.[2] Quantitative differences in susceptibility were found in the disease group as compared to normal individuals. Erythrocytes from individuals with coryza also demonstrated increased susceptibility to phagocytosis. That the modified cell may play a role when acute infections precipitate crises in chronic hemolytic states has been suggested.[3]

The modified red blood cell does have a shortened survival, as was shown previously by the following: (1) Sensitized red blood cells in the rabbit[4] and

humans,[5, 6] (2) trypsin-treated red blood cells in the rabbit (this shortened survival lengthened with splenectomy),[7] and (3) virus-treated red blood cells in the intact dog.[8]

The present study was designed to evaluate the survival of autologous erythrocytes subsequent to virus treatment in vitro in normal and splenectomized rabbits.

Materials and methods

Animals. Young adult white rabbits of both sexes were obtained commercially.

Viruses. Allantoic fluid containg Newcastle diseases virus (NDV) with a hemagglutinin titer of 1:2,048 was used. The virus originally was isolated from a chicken with a fatal case of the disease and had been passaged many times in fertile hen eggs. Influenza virus was obtained with hemagglutinin titers of 1:512 and 1:1,024.

Virus treated erythrocytes. Blood was obtained from rabbits by cardiac puncture and collected in tubes containing sodium versenate. The red blood cells were separated by centrifugation and washed 3 times in phosphate buffered saline. Virus treatment was carried out by incubating one part packed red blood cells with one part virus material at 37° C. for 2 to 4 hours. The treated cells were then washed 4 times in saline, reconstituted with original plasma,

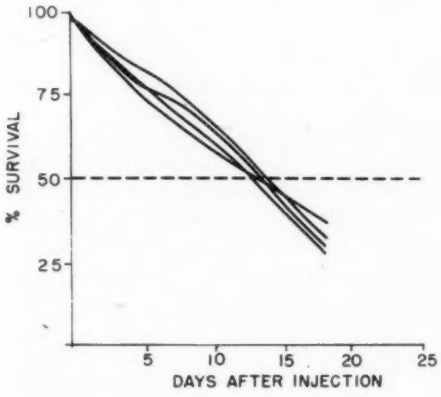

Fig. 2. The survival of erythrocytes tagged in vivo with radiochromium in 4 normal rabbits. The 50 per cent survival time was essentially the same as that obtained with in vitro tagging methods.

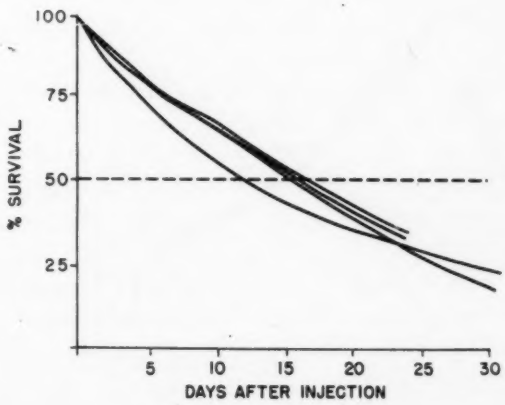

Fig. 3. The survival of normal erythrocytes tagged in vitro with radiochromium in 4 splenectomized animals. The 50 per cent survival time was 16, 16, 12, and 15 days, as compared with a mean of 12 days for normal animals.

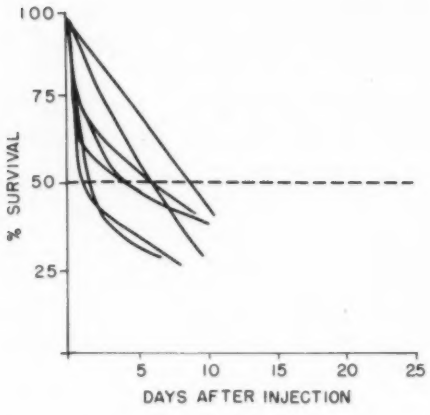

Fig. 4. The survival of influenza virus–treated erythrocytes in 7 normal rabbits. The 50 per cent survival was from less than 24 hours to 9 days.

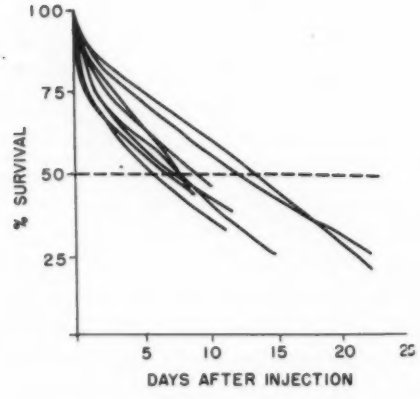

Fig. 5. The survival of influenza virus–treated erythrocytes in 8 splenectomized rabbits. Half times of survival were as follows: 6, 9, 11, and 13 days in single animals, and 8 days in 4 animals.

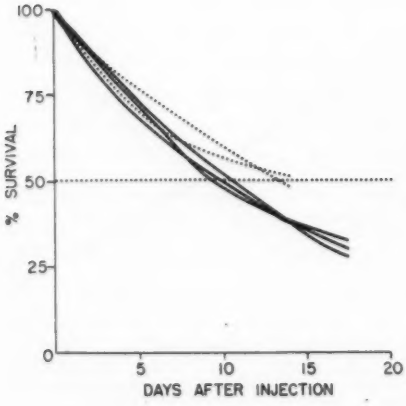

Fig. 6. The survival of erythrocytes treated with normal allantoic fluid in 3 rabbits (solid lines) and of erythrocytes washed 10 times in isotonic saline in 2 rabbits (broken lines).

and added to the special acid citratedextrose* solution. The cells were tagged, centrifuged, and then washed 3 times. The washed, packed cells were diluted in saline and injected via a marginal ear vein.

Erythrocyte survival. The following two methods of tagging erythrocytes in vitro were used for the survival studies: (1) Whole blood was added to special A.C.D. solution and subsequently tagged with 100 to 150 μc sodium chromate (CR^{51}) for periods of 15 minutes to 1 hour at room temperature. Following incubation, 50 mg. of ascorbic acid was added to the mixture to reduce unbound chromium. The blood-A.C.D. mixture was then injected into the donor rabbit via a marginal ear vein. The standard, representing 100 per cent survival, was obtained 24 hours after injection. Additional blood samples were obtained in 3 ml. amounts 2 to 3 times weekly. The percentage of radioactivity of subsequent samples was calculated from the standard based on the hematocrit value. (2) The alternate tagging procedure utilized in animals receiving virus-treated cells consisted of the injection of washed red blood cells labeled with CR^{51} in place of whole blood. Standard counts representing 100 per cent survival were determined 10 to 20 minutes after injection of tagged cells. Subsequent 2 ml. samples were taken via a marginal ear vein twice during the first 24 hours and 3 times weekly thereafter.

In vivo tagging of erythrocytes was accomplished by the intravenous injection of 100 to 150 μc of sodium chromate diluted in A.C.D. solution. Samples of blood for counting were obtained beginning 24 hours after injection. Plasma counts were determined in conjunction with whole blood counts until the radioactivity of the plasma was found to be negligible.

Controls were carried out to determine the effects of procedures and normal allantoic fluid on normal erythrocytes employing the same techniques used for the virus studies.

Results

I. Survival in normal rabbits of normal erythrocytes.

A. IN VITRO TAGGED RED BLOOD CELLS. A composite of the data of the survival studies of normal erythrocytes in 17 normal animals is shown in Fig. 1. To facilitate the study, the radioactivity of blood samples was determined for a period of time to include or extend slightly beyond the apparent 50 per cent survival time. A limited number of observations were made to determine the total length of time that the cells survived. The apparent half time of survival in individual animals ranged between 8 and 17 days, with a mean of 12 days. Those studies, when carried to completion, showed a survival of between 55 and 60 days.

B. IN VIVO TAGGED RED BLOOD CELLS. Fig. 2 shows the survival of erythrocytes in 4 animals tagged in vivo by the intravenous injection of radiochromium. The mean 50 per cent survival was found to be essentially the same as in animals tagged by in vitro methods.

II. Survival in splenectomized rabbits of normal erythrocytes tagged in vitro. The effect of splenectomy on the survival of normal erythrocytes in 4 animals is shown in Fig. 3. It appears that the 50 per cent survival time was extended slightly; the values being 16, 16, 12, and 15 days. The percentage of cells surviving appeared to be greater throughout the 24 and 35 days in which determinations were made.

III. Survival in normal rabbits of virus-treated erythrocytes tagged in vitro. The survival of erythrocytes exposed to the action of influenza virus in vitro was determined in 7 normal rabbits. There appeared to be considerable variation

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in the survival characteristics of treated erythrocytes in individual animals; however, the over-all effect was one of shortening the life span. The range of apparent 50 per cent survival in this group of animals was 1½ to 9 days (Fig. 4). A similar experiment, using NDV and trypsin as the modifying agents in 2 and 4 animals, respectively, resulted in an apparent half-life of 24 hours or less.

IV. Survival in splenectomized rabbits of influenza virus–treated erythrocytes tagged in vitro. The effect of splenectomy on survival of influenza virus–treated erythrocytes is illustrated in Fig. 5. This shows an increase in the 50 per cent survival time in 8 splenectomized animals when compared with the survival of virus-treated cells in nonsplenectomized animals illustrated in Fig. 4. Apparent half times of survival in 8 determinations in the splenectomized animals were as follows: 6, 9, 11, and 13 days in single animals and 8 days in 4 animals.

V. The effects of multiple washing and normal allantoic fluid on erythrocyte survival. Fig. 6 shows the survival times of normal erythrocytes treated with normal allantoic fluid in 3 rabbits. The procedures were the same as those used in treating erythrocytes with allantoic fluid containing virus. The survival of these cells was within the normal range.

In addition, the broken lines in Fig. 6 indicate the survival times of erythrocytes in 2 rabbits whose cells were washed in isotonic saline a total of 10 times. Again the erythrocytes survived normally in the autologous animal.

Discussion

The basic mechanisms involved in destruction of erythrocytes have been approached through the study of the following: (1) Various agents capable of modifying the red blood cells[9]; (2) demonstrating these modifications by serologic methods[10, 11]; (3) the susceptibility of the modified red blood cells to phagocytosis by tissue culture macrophages[12]; (4) in vivo survival of modified red blood cells in animals; and (5) the clinical counterparts of these phenomena.[3]

The present report deals with correlating the in vitro studies with observations in the intact animal. It utilizes the well-established method of tagging erythrocytes with radioactive chromium for study of their survival. This technique has been used for normal and virus-treated erythrocytes in normal and splenectomized animals.

The survival of normal rabbit erythrocytes has been studied by a number of investigators using a variety of techniques[4, 13-15] and the values reported agree well with determinations made during this investigation. Thus the values of 12 days for the apparent half-life and 55 to 60 days for the maximum survival of normal rabbit erythrocytes are assumed to be valid indices of comparison for the study of the effects of virus and enzyme treatments on the erythrocyte survival.

Results obtained by the method of tagging and injecting whole blood and the method of tagging and returning only the red cells to the circulation produced comparable results. The intravenous injection of radiochromium to affect in vivo erythrocyte labeling resulted in similar findings.

Studies carried out on tagged erythrocytes in splenectomized animals also indicated a normal pattern of red cell survival. There appeared to be a slight increase in the apparent 50 per cent survival to the upper limits of normal which persisted throughout the 24 and 35 day periods of observation. These findings may be the result of a physiologic difference in the red cell population in which a greater number of young cells are represented in the previously splenectomized animal.

Changes produced on red blood cells resulting from the action of the hemagglutinating viruses, influenza and Newcastle disease, are reflected in (1) increased fragility,[16] (2) change in electrophoretic mobility,[17, 18] (3) refractiveness to further virus action,[19] (4) change in antigenicity,[11, 20] and (5) increased susceptibility to phagocytosis.[9] The present study also indicates, as might be expected, a shortened survival after the virus-treated erythrocytes are returned to the circulation of the donor animal. Differences encountered in the survival of erythrocytes in individual animals were striking; however, in each case the survival of the cells was decreased. It appeared that Newcastle disease virus and trypsin produced a more deleterious effect on the rabbit erythrocytes than did influenza virus, as judged by the greatly reduced survival time. Evidence of the increased destructive effects of these agents was also noticeable in the virus and trypsin red cell mixture following incubation, in which considerable hemolysis occurred. Erythrocytes treated with normal allantoic fluid showed normal survival following reinjection. Also, the present studies and those of other investigators[21, 22] suggest that multiple washing of erythrocytes does not significantly affect their survival.

That the virus-treated erythrocyte is vulnerable to destruction by phagocytic cells in tissue culture has been shown. Furthermore, the sites of red blood cell destruction in patients and animals receiving tagged red blood cells which are defective or incompatible have been studied and the spleen and liver are the main foci of increased radioactivity. On the basis of these findings the experiment using influenza virus–treated erythrocytes was repeated in rabbits previously splenectomized. It is suggested that the removal of this source of reticuloendothelial tissue permitted the cells to remain in the circulation for a longer period of time, although again the factor of individual differences was encountered. Preliminary studies on the survival of erythrocytes treated with Newcastle disease virus in splenectomized rabbits indicate that the survival of the cells is not significantly altered. Additional studies are needed to substantiate this finding.

The clinical counterpart of the shortened survival of the virus-modified red blood cell is seen with the influence of acute infection such as coryza in precipitating acute hemolytic crises in sickle-cell disease.[3]

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DNA synthesis in circulating blood leukocytes labeled in vitro with H^3-thymidine

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Blood specimens from patients with various hematologic disorders or virus diseases as well as from a group of patients with various diagnoses but normal hemograms were incubated in vitro with H^3-thymidine. Labeling of circulating deoxyribonucleic acid synthesizing cells was detected in autoradiograms of leukocyte smears. Among 28 patients with normal hemograms, small numbers of labeled mononuclear cells, 5 to 10 per 10,000 total leukocytes, capable of labeling were found. Significantly increased numbers of labeled mononuclear cells were seen in patients with either various viral infections, multiple myeloma, or chronic leukemia. No increase was seen in polycythemia vera. Labeled myelocytic or leukemic cells were seen in myelocytic and acute leukemia.

Tritiated thymidine (H^3TDR)† cell labeling has become an increasingly useful technique for evaluating cellular proliferation. This tritium-labeled deoxyriboside is incorporated into new deoxyribonucleic acid (DNA) of proliferating cells in vivo or during in vitro incubation. The resulting intranuclear H^3DNA label is detected by microautoradiographic methods. Labeled cells are those which were synthesizing DNA during H^3TDR availability and are considered to be destined for mitosis. Mature cells, incapable of proliferation, do not incorporate this label.

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Supported by the United States Public Health Service and by the United States Atomic Energy Commission.

Received for publication Feb. 24, 1961.

*In addition, Dr. G. Brecher, National Institute of Health, Bethesda, Md., Dr. L. Meyer, South Nassau Hospital, Rockville Center, N. Y., and Drs. T. Vietti and J. Whitaker, Department of Pediatrics, Southwestern Medical School, University of Texas, Dallas, Texas, collaborated in portions of these studies.

†Schwarz Bioresearch Inc., Mt. Vernon, N. Y.

Table I. Comparison of labeling indices (labeled cells per 10,000 leukocytes counted) using different film types and exposure times

Patient	*Kodak NTB-3 dipping emulsion, 7 day exposure*	*Kodak AR-10 stripping film, 14 day exposure*
H. R.	40	50
M. S.	10	0
G. M.	0	10
L. C.	15	23
A. D.	500	580
J. A.	200	170

In vitro incubation of blood leukocytes with H^3TDR has demonstrated the presence of small numbers of circulating labeled mononuclear cells in specimens obtained from 6 normal adult subjects.[1] Significantly increased numbers of labeled proliferative cells were detected in blood from patients with acute infectious mononucleosis, various infections, and leukemias, and also in blood from patients exposed to total body radiation.[2-5] The fate and function of these circulating proliferative cells remain unclear.

The present investigation is a further evaluation of the proliferative potential of blood leukocytes from patients with varied diseases. In vitro labeling of blood leukocytes with H^3TDR was evaluated in patients with white cell counts abnormal due to either hematologic disorders or infections. In contrast, a series of hospitalized patients with normal W.B.C. counts and differentials was similarly studied.

Materials and methods

In general, the in vitro incubation mixtures consisted of 2 ml. of heparinized venous blood and 0.1 ml. normal saline containing 1 μc of H^3TDR of high specific activity (1.9 c per millimole); the mixtures were incubated in siliconized test tubes. After mixing, the tubes remained for 1 hour at room temperature. After this incubation period they were centrifuged at 1,800 r.p.m. for 5 minutes. The buffy coats were aspirated and placed in siliconized test tubes and 2 to 3 drops of supernatant plasma were added to resuspend the concentrated leukocytes. Leukocyte smears were made, dried rapidly, and fixed in methanol. Autoradiograms were made using Kodak AR-10 stripping film or Kodak NTB-3 liquid emulsion. The autoradiograph slides were exposed routinely for 1 to 2 weeks; developed, washed, and stained with Giemsa or May-Grünwald stains. The labeling index (l.i.) was obtained by counting the number of H^3-labeled leukocytes in a total of at least 1,000 leukocytes (cells with 5 or more grains over their nuclei were considered labeled). The gross labeling index is the computed number of labeled cells per 10,000 nucleated cells, independent of cell type. If no labeled cells were seen during the usual cell count, then the autoradiograms were scanned for at least 10 minutes more, or until at least one labeled cell was found. Absence of labeled cells in these autoradiograms is not considered erroneous because in all instances preparations processed simultaneously had labeled cells.

In some instances duplicate leukocyte smears were treated with dipping emulsion or stripping film and exposed for varying lengths of time in order to determine the effects of film type and varied exposure. With either film, and for exposures of 1 or 2 weeks, the labeling indices were not significantly different. In Table I is shown the close agreement in the labeling index between autoradiograms exposed with dipping emulsion for 7 days and those exposed with stripping film for 14 days. Longer exposure increased the grain count

Table II. Gross labeling index in patients with nonhematologic disorders

Patient	*Age in years*	*Diagnosis*	*Date labeled (1960 unless noted otherwise)*	*Leukocytes*		*Gross labeling index (per 10,000 W.B.C.)*
				Total counted	*Amount labeled*	
1. A. B.	52	Rheumatic heart disease	March 24	1,000	0	0
2. J. G.	64	Arteriosclerotic heart disease	Jan. 8	5,700	4	7
3. R. J.	31	Aortic aneurysm; epilepsy	Jan. 8	3,000	3	10
4. J. P.	62	Hypertensive heart disease	Jan. 8.	3,000	4	13
5. H. D.	64	Hypertensive heart disease	March 24	3,000	6	20
6. J. R.	32	Diabetes mellitus; peptic ulcer	Jan. 15	3,000	3	10
7. B. T.	26	Tonsillitis; peptic ulcer	Jan. 15	3,000	4	13
8. H. P.	21	Diabetes mellitus	Jan. 15	6,000	6	10
9. W. B.	32	Dermatophytosis	Jan. 15	3,000	4	13
10. E. P.	41	Hepatitis	Feb. 19	3,000	0	0
11. A. G.	36	Acute tuberculosis	Jan. 29	3,000	3	10
12. A. C.	58	Tuberculosis	Jan. 26	3,000	1	3
13. J. R.	32	Normal man	Feb. 1	3,000	1	3
			Feb. 4	3,000	4	13
14. M. M.	32	Normal woman	Feb. 1	3,000	0	0
15. H. R.	47	Toxic goiter	Feb. 12	3,000	0	0
16. E. E.	35	Recovery from infectious mononucleosis	March 3	3,000	0	0
17. L. C.	40	Myotonia dystrophica	March 21	3,000	0	0
			March 22	3,000	1	3
18. J. T.	71	Cerebral vascular accident	March 24	3,000	0*	0
19. W. B.	61	C.N.S. syphilis; diabetes mellitus	March 24	3,000	6	20
20. E. P.	45	Hypertensive heart disease	Jan. 8	4,500	0	0
21. O. H.	49	Alcoholism	Feb. 19	3,000	0	0
22. F. R.	63	Peptic ulcer	March 24	2,000	0	0
23. G. M.	28	Psychoneurosis	March 24	2,000	3	15
24. M. S.	61	Arteriosclerotic heart disease	March 24	3,000	0	0
			March 24	2,000	2	10
25. J. F.	35	Peptic ulcer	March 24	3,000	0	0
26. G. M.	61	Peptic ulcer	March 24	3,000	3	10
			March 24	3,000	0	0
27. C. N.	30	Cortical atrophy	Jan. 8	3,000	1	3
28. S. M.	36	Ankylosing spondylitis	Jan. 8	3,000	0	0
29. W. O.	42	Rheumatoid arthritis	March 24	3,000	0	0
30. L. H.	65	Myxedema	Jan. 11	3,000	0	0
31. A. M.	45	Thyrotoxicosis	Dec. 3, 1959	3,000	0	0
32. F. W.	49	Hypertension	Jan. 8	5,000	0	0
33. J. J.	61	Amyotrophic lateral sclerosis	Jan. 8	3,000	5	17
34. C. L.	64	Hypertension	Jan. 8	3,000	0*	0
35. T. L.	43	Chronic alcoholism	Jan. 8	4,500	0	0
36. E. K.	64	Pulmonary emphysema	Jan. 8	3,000	0	0
37. J. M.	47	Postoperative lung cancer	Jan. 8	4,000	0	0
38. C. R.	72	Arteriosclerotic heart disease	Jan. 8	3,000	2	7

Table II. Cont'd

Patient	*Age in years*	*Diagnosis*	*Date labeled (1960 unless noted otherwise)*	*Leukocytes*		*Gross labeling index (per 10,000 W.B.C.)*
				Total counted	*Amount labeled*	
39. A. B.	64	Carcinomatosis	Jan. 8	3,000	0*	0
40. J. M.	40	Neurosyphilis	Nov. 25, 1959	7,500	8	11
41. K. S.	35	Hyperthyroidism	Dec. 3, 1959	3,000	5	17
42. C. S.	44	Cancer of the stomach	Nov. 25, 1959	4,000	4	10
43. J. B.	41	Psoriasis; rheumatoid arthritis	Jan. 8	3,000	2	7
44. J. P.	32	Metastatic lung cancer	Feb. 26	1,000	1	10
		Metastatic lung cancer	March 15	3,000	0	0
		Metastatic lung cancer	March 16	1,000	1	10
		Metastatic lung cancer	March 17	3,000	0	0
45. C. H.	39	Hydradenitis	Feb. 25	1,000	0	0
		Hydradenitis	March 16	1,000	0	0
		Hydradenitis	March 17	3,000	3	10
		Hydradenitis	March 18	3,000	3	10
46. E. C.	58	Cor. pulmonale	March 24	2,000	1	5
47. A. C.	64	Cancer of prostate	March 24	2,000	1	5
48. H. S.	32	Psychoneurosis	March 24	2,000	0	0

*Labeled cells seen only on scanning after a 3,000 W.B.C. count.

per cell, but did not significantly affect the labeling index. Stripping film yields somewhat smaller and sharper grains than does the dipping emulsion.

Results

I. Patients with nonhematologic disorders. Table II describes a group of male patients with nonhematologic disorders in which DNA synthesis in circulating cells was assessed by in vitro H^3TDR labeling. These patients had white cell counts ranging between 5,000 and 10,000 per cubic millimeter with essentially normal differential counts. The total number of leukocytes counted in each autoradiogram, the number of labeled cells found, and the gross labeling index are shown. In some patients cell labeling was repeated on different days. A total of 58 in vitro incubations with H^3TDR were performed on 48 patients. The gross labeling index of these patients ranged from zero to 28. In 30 instances circulating cells capable of being labeled were seen, while in 28 instances no labeled cells were found. The additional 10 minute scan of these negative autoradiographs yielded only 3 slides in which labeled cells subsequently were found. The average gross labeling index of the 30 positive autoradiograms was 10. The over-all average gross labeling index was 5 per 10,000, or 0.05 per cent. Patient ages ranged from 21 to 72 years. The labeling indices of these adult patients did not vary with age. Repeat labeling index determinations on the same patient were done in a few instances; in these patients the labeling index was seen to be relatively constant (Patients Nos. 13, 17, 24, 26, 44, and 45).

The types of labeled cells and their relative frequency corresponded to those described previously.[1, 2] All were mononuclear in type, some resembling lympho-

cytes ("atypical" lymphocytes) and some resembling monocytes. A satisfactory differential count of the labeled cells could not be done because their population was very small and precise identification was difficult. Labeling indices obtained at 10 A.M., 4 P.M., 10 P.M., and 4 A.M. on 4 patients of this group (and 2 leukemic patients) showed no detectable diurnal variation.

II. Patients with viral diseases. In vitro labeling of blood specimens with H^3TDR was done in 14 children and 4 adults who had viral infections. Results are shown in Table III. Difficulties in obtaining adequate follow-up studies of the children prevented definite diagnosis to be made in some (indicated by question marks in the table). Markedly increased numbers of circulating proliferating cells were seen in many of these children. One child with streptococcal infection who failed to show increased labeling is included. All labeled cells appeared to be of the same types as those found labeled in normal blood.

III. Patients with hematologic disorders. The results from patients with various hematologic disorders are shown in Table IV. Their diagnoses and other pertinent information are given with the results of the labeling procedures; additional information and comment not included in the table are given below.

Of the first 3 patients (leukemoid reactions), the first 2 had leukocytosis with differentials indicating over 90 per cent mature granulocytes. No labeled myeloid precursor cells were found. Patient No. 3 manifested an initial differential count with myelocytes, metamyelocytes, and small numbers of circulating orthochromatic normoblasts. The leukocyte alkaline phosphatase activity was markedly elevated (95 per cent). Labeled cells were seen in distinctly increased numbers, consisting of promyelocytes and myelocytes as well as labeled mononuclear cells of types seen in normal peripheral blood. Patients Nos. 4 to 6 had myeloid metaplasia, confirmed by liver and bone marrow biopsy and/or autopsy. Both Patients Nos. 4 and 5 had circulating nucleated erythrocytes (Patient No. 4 had 21 erythroid precursors per 100 total nucleated cells). An increase in the number of labeled cells, with labeled myelocytes and types of mononuclear cells seen labeled normally, was observed in all patients. A few labeled polychromatophilic normoblasts were seen on smears from Patient No. 4. In Patients Nos. 7 to 13 (polycythemia vera), no increases in the number of labeled cells were noted, with one exception: Patient No. 14 (aplastic anemia) was severely leukopenic, with a differential revealing 74 per cent small lymphocytes, 6 per cent monocytes, and 20 per cent granulocytes. Increased numbers of normally labeled mononuclear cell types were seen.

Patients Nos. 15 to 18 had multiple myeloma with elevated serum globulins and/or Bence-Jones proteinuria, and typical changes of myeloma in their bone marrow. Small numbers of atypical plasmablasts and plasma cells were seen circulating in some of these patients. The increased labeling index noted was principally due to increased numbers of mononuclear cell types found normally. Labeled myeloma cells were not seen. Patient No. 19 was shown to have macroglobulinemia by ultracentrifuge studies. The marrow aspirate contained increased numbers of mast cells and plasmacytoid lymphocytes compatible with Waldenström's macroglobulinemia.[6] At the time of the labeling a lymphocytosis was present, and the patient had been receiving 6 mg. of chlorambucil daily for

Table III. Gross labeling index of patients with virus disease

Patient	*Age*	*W.B.C.*	*Mononuclear cells* (%)*	*Diagnosis*
1. J. B.	19 months	9,650	48	Measles?
2. B. W.	4½ years	—	56	Measles? Echo virus?
3. J. H.	3 years	5,400	45	Measles
4. B. M.	16 months	8,000	45	Measles
5. D. N.	1½ years	5,000	80	Measles
6. W. B.	11 months	—	38	Measles Pneumonia
7. P. S.	9 months	17,500	50	Measles
8. R. C.	9 years	13,700	23	Mumps?
9. W. P. B.	30 years	12,250	48	Mumps
10. A. H.	48 years	—	55	Mumps
11. K. W.	34 years	—	59	Mumps
12. B. H.	5 years	16,750	35	Chickenpox Pneumonia
13. T. P.	58 years	7,100	33	Chickenpox
14. H. M.	4 years	8,400	42	Herpes stomatitis
15. K. J.	6 months	16,000	72	Infectious mononucleosis
16. R. H.	3 years	7,900	91	Fever of unknown origin
17. S. McG.	2 years	14,800	22	Streptococcus tonsillitis
18. D. G.	22 months	75,000 20,000	92	Infectious lymphocytosis

*This includes typical and atypical monocytes as well as leukocytes.

the previous 6 days. The increased number of labeled cells found was principally due to cells that label normally.

The remaining patients (Nos. 20 to 50) had leukemia or lymphosarcoma, as indicated in the table. All diagnoses were made on the basis of blood and marrow smears, biopsy, and clinical course. On many patients multiple samples on different dates were taken; serial blood count and labeling index changes following chemotherapy will be reported separately.[7] The values given are representative. The therapy received and other information is given under "Comment" in the table.

Discussion

The present findings confirm and extend our previous observations that small numbers of mononuclear cells capable of DNA synthesis are present normally in the peripheral blood, and that the number of such cells increases with some disease processes.[1-5] The patients described with nonhematologic disorders

Date labeled (1960)	*Cells counted*	*Gross labeling index (per 10,000 W.B.C.)*	*Comments*
Feb. 10	1,000	370	Symptoms Feb. 7, 1960
Feb. 26	1,000	310	Cough and fever, 4 days
Feb. 16	1,000	60	Cold and cough, 5 days
Feb. 10	1,000	0	Onset Jan. 31, 1960
Feb. 20	1,000	80	Sick off and on, 3 weeks; current symptoms, 2 days
April 14	1,000	30	8 days symptomatic
April 4	1,000	190	3 days symptomatic; 4-1-Koplik spots
Feb. 12	1,000	0	Symptoms 1 day
April 29	2,000	120	9 days illness
April 15	3,000	90	13 days illness
March 24	1,000	220	10 days illness
April 15	1,000	20	
Feb. 18	500	120	Symptoms 3 days
May 5	1,000	60	4 days illness
Feb. 24	1,000	10	Symptoms started Feb. 16
Feb. 21	500	280	Pneumonia Feb. 19, 1960; hepatosplenomegaly and rash Feb. 21, 1960
April 4	1,000	190	Symptoms 1 day
May 5	1,000	10	Symptoms 2 days
Sept. 1	1,000	250	Heterophile negative;
Sept. 14	1,000	180	95% small mature lymphocytes

who had normal leukocyte counts represent an obviously mixed population of diseases. Of significance is the absence of increased labeling seen in 5 patients with malignancy (Table II, Patients Nos. 37, 39, 42, 44, and 47) in whom circulating cancer cells probably were present.[8] Additional studies designed to detect labeling in these circulating cancer cells, using recent methods for their concentration from blood,[8] appear to be indicated. In view of the very low label index of various tumors labeled in vivo[9] and in vitro[10, 11] with H^3TDR, a low percentage of labeling in circulating malignant cells might be anticipated. A direct correlation between venous invasion by malignancy and the presence of free tumor cells in the venous effluent from the tumor area has been shown.[8] It is stated that probably 99 per cent of all tumor cells reaching the circulation fail to survive. Recent studies with H^3TDR have given further support to this concept.[11]

It is apparent from Table III that some children and adults manifest large increases in circulating DNA synthesizing mononuclear cells during the acute

Table IV. Labeling index in patients with blood dyscrasias

Patient	*Age in years*	*White cell count*	*Diagnosis*	*Date labeled*	*Total cells counted*	*Gross labeling index (per 10,000 W.B.C.)*	*Comments*
1. M. A.	71	34,100	Leukemoid reaction	March 4, 1960	3,000	0	Diabetic with gangrene of toe
2. L. C.	68	52,100	Leukemoid reaction	Feb. 29, 1960	2,000	15	Periarteritis nodosa
3. S. L.	65	103,400	Leukemoid reaction	Feb. 11, 1960	2,000	180	Perinephric abscess
		11,200		May 5, 1960	1,000	30	Perinephric abscess
4. R. T.	67	5,600	Myeloid metaplasia	Jan. 19, 1960	3,000	68	
		6,600		March 22, 1960	2,000	85	
5. R. J.	64	30,250	Myeloid metaplasia	Jan. 20, 1960	2,000	225	
6. R. Z.	43	16,900	Myeloid metaplasia	Sept. 28, 1959	5,000	400	
7. K. L.	70	7,300	Polycythemia vera	Oct. 12, 1959	1,000	100	
8. J. S.	65	19,700	Polycythemia vera	May 15, 1958	1,000	30	
9. R. C.	50	10,600	Polycythemia vera	Oct. 18, 1960	1,000	30	
10. E. M.	72	9,400	Polycythemia vera	May 5, 1958	1,000	0	In remission
11. G. S.	45	13,200	Polycythemia vera	Jan. 27, 1960	3,000	0	In remission, hematocrit 46
12. J. B.	47	12,300	Polycythemia vera	May 5, 1960	3,000	0	Hematocrit 58
13. D. T.	41	6,650	Polycythemia vera	April 20, 1960	6,650	1	1,650,000 platelets
14. G. W.	65	1,300	Aplastic anemia	Jan. 19, 1960	2,000	60	† Chloramphenicol toxicity
15. E. B.	44	3,600	Multiple myeloma	Aug. 8, 1958	1,000	80	
16. N. G.	66	4,500	Multiple myeloma	Dec. 10, 1958	1,000	30	
17. C. M.	67	4,500	Multiple myeloma	Dec. 10, 1958	1,000	0	
18. L. C.	49	28,000	Multiple myeloma	March 24, 1960	2,000	60	
		23,000		March 25, 1960	2,000	45	
		18,000		March 28, 1960	2,000	35	
19. E. B.	68	3,350	Waldenström's macroglobulin-	Feb. 19, 1960	2,000	35	Leukoran, 6 mg. 4 times daily
		4,700	emia	Feb. 24, 1960	2,000	75	
20. H. R.	64	18,400	Chronic myelocytic leukemia	Feb. 16, 1960	2,000	65	With aregenerative anemia
		*		Feb. 24, 1960	2,000	20	With aregenerative anemia
		19,950		March 16, 1960	2,000	45	With aregenerative anemia
		*		March 17, 1960	2,000	20	With aregenerative anemia
		*		March 18, 1960	2,000	50	With aregenerative anemia

		*		March 17, 1960	2,000	20	With aregenerative anemia
				March 18, 1960	2,000	50	With aregenerative anemia

21. A. D.	41	114,000	Chronic myelocytic leukemia	Jan. 18, 1960	2,000	540	
		100,000		Jan. 20, 1960	1,000	570	
22. J. K.	42	123,000	Chronic myelocytic leukemia	Jan. 25, 1960	5,000	300	
23. L. W.	68	111,500	Chronic myelocytic leukemia	Oct. 6, 1958	5,000	500	
24. A. W.	32	87,000	Chronic myelocytic leukemia	Jan. 25, 1958	1,000	180	
25. E. F.	40	3,000	Chronic myelocytic leukemia	Aug. 26, 1958	3,000	47	
26. R. M.	50	195,000	Chronic myelocytic leukemia	Sept. 18, 1957	1,000	1,610	In blastic crisis
27. M. M.	59	19,700	Chronic myelocytic leukemia	Oct. 12, 1959	1,000	10	
28. H. E.	48	130,000	Chronic myelocytic leukemia	Sept. 18, 1958	5,000	268	
29. A. W.	73	125,400	Chronic lymphocytic leukemia	Dec. 4, 1958	3,000	66	
30. M. L.	70	19,000	Chronic lymphocytic leukemia	Oct. 20, 1958	5,000	40	
31. W. S.	61	191,000	Chronic lymphocytic leukemia	Jan. 25, 1958	3,000	16	
32. J. K.	46	2,000	Chronic lymphocytic leukemia	June 14, 1958	3,000	7	
33. R. F.	53	9,900	Chronic lymphocytic leukemia	May 26, 1958	3,000	20	
34. B. K.	47	102,000	Chronic lymphocytic leukemia	May 24, 1958	3,000	0	
35. E. M.	61	331,000	Chronic lymphocytic leukemia	June 5, 1958	3,000	0	
36. P. A.	38	3,800	Lymphosarcoma	Oct. 7, 1959	1,000	90	
37. C. C.	69	31,400	Lymphosarcoma	Jan. 6, 1960	1,000	40	
38. C. S.	74	20,700	Lymphosarcoma	Oct. 8, 1959	1,000	200	
39. H. M.	28	3,200	Acute myelocytic leukemia	April 9, 1958	3,000	0	
40. M. K.	60	166,000	Acute myelocytic leukemia	Aug. 12, 1958	3,000	420	
41. C. W.	32	12,200	Acute myelocytic leukemia	April 8, 1958	3,000	40	
42. B. K.	69	8,000	Acute myelocytic leukemia	Aug. 12, 1958	3,000	187	
43. M. M.	51	7,900	Acute myelocytic leukemia	Jan. 25, 1958	1,000	0	
44. I. N.	24	3,500	Acute myelocytic leukemia	Dec. 18, 1959	1,000	10	
45. J. G.	51	42,700	Acute myelocytic leukemia	May 4, 1960	1,000	330	
				May 5, 1960	1,000	270	
46. J. A.	39	5,950	Acute myelocytic leukemia	Dec. 16, 1959	3,000	140	
47. G. M.	71	7,500	Acute stem cell leukemia	March 25, 1960	1,000	310	
48. J. B.	16	150,000	Acute stem cell leukemia	April 21, 1960	1,000	1,410	
49. C. B.	4	35,700	Acute stem cell leukemia	March 17, 1960	1,000	810	
50. P. F.	62	24,000	Acute stem cell leukemia	March 1, 1960	1,000	530	

*Leukocyte count not done.

phases of various viral and presumed viral illnesses. Furthermore, the present data suggest that during recovery the labeling index falls to lower (presumably normal) levels. An increased labeling index was not found in a small series of children tested who had various diagnoses with essentially normal white counts.* Bond and associates[2] and Gavosto and colleagues[4] previously have shown similar increased labeling in early infectious mononucleosis which returns to normal during recovery. One infant with infectious lymphocytosis (Table III, Patient No. 18) had a high labeling index which closely resembled that of a patient with infectious mononucleosis; the heterophile was repeatedly negative. It is reasonable, however, to suspect that viral infection might induce changes in the proliferative capacity of leukocytes. The increased labeling index seen in some of our patients appears to corroborate this possibility.

Bierman and co-workers[13] have reported remission from acute leukemia in children during the course of varicella and some bacterial infections. One can speculate that, whatever the mechanism involved, numbers of nonleukemic normal cells capable of further DNA synthesis increased with the viral or bacterial infection in these children. The capacity for further division of these cells may have resulted in temporary successful competition with the leukemic population for some essential metabolite, with a resulting remission of the leukemic process. Studies to evaluate the effect of viral and other infections in acute leukemia as studied with H^3TDR are in progress.

The different hematologic disorders reported here fell into 3 categories with regard to the numbers and type of cells that label: those with no increase, those with an increase in the types of cells that label normally, and those with an increase due to labeling of cells characteristic of the disease. In most patients with polycythemia vera, no increase over normal in the labeling index was observed, and the cells that labeled were those seen normally. Most of these patients were not severely symptomatic from their disease. Patients with leukemoid reaction, aplastic anemia, multiple myeloma, and Waldenström's macroglobulinemia all manifested increased labeling indices, but predominantly because of an increase in the numbers of labeled cells of types normally present. Only rarely were labeled cells of an abnormal type seen. In acute leukemia, chronic myelocytic leukemia, and myeloid metaplasia the vast majority of labeled cells were myeloid precursor elements or leukemic cells. It is possible that the number of labeled cells of types found normally was also increased; however, these types contributed little to the labeling index. The highest labeling index was noted in the acute childhood leukemias. In one instance, 14.1 per cent of all nucleated blood cells were labeled in a child with acute leukemia (Table IV, Patient No. 48).

In general, in hematologic diseases associated with hemopoietic irritation or stimulation (leukemoid reactions, myeloid metaplasia, multiple myeloma, and some myelocytic leukemias), labeled myeloid and/or erythroid precursor forms appeared in the blood. Increased numbers of labeled "leukemic cells" in the

*Unpublished observations of the authors.

blood were seen in most myelocytic leukemias[2, 3, 14]; no increases have been seen in chronic lymphocytic leukemia.

As indicated from these present studies, the numbers of these cells in the blood might be increased under certain conditions. A transient increase in their numbers is present following total or partial body irradiation.[5] That shielding of the spleen or marrow, or injection of preparations of these organs can be protective against radiation death is well known. Parabiosis[15] or differential partial body exposure with time[16] affords similar protection, pointing to transfer of a protective moiety via the blood stream. Transfer of white cells from mice with a leukemoid reaction in which H^3TDR blood cell labeling was demonstrated afforded significant radiation protection.[17] Infused normal peripheral blood can induce marrow graft rejection and the "runt disease" in irradiated animals.[18, 19] These data taken collectively would provide strong argument that either cells exist in the peripheral blood that are pluripotential, or that peripheral blood, by some unknown mechanism, can induce accelerated proliferation of cells under some conditions in a recipient host. It is similarly possible that these circulating proliferative cells may be associated normally with regulation of at least some types of proliferative cell compartments in the body, and that the number of these circulating proliferative cells may be increased under conditions demanding a greater than normal degree of proliferation.

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The electrophoretic mobility of the plasma vitamin B_{12}-binding protein of man and other vertebrate species at pH 4.5

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The plasma vitamin B_{12}-binding protein ($B_{12}BP$) of man was soluble in sulfosalicylic acid and migrated anodally when it was electrophoresed at pH 4.5 on a starch block. The plasma $B_{12}BP$ of the Rhesus monkey, guinea pig, and rat was only minimally soluble in sulfosalicylic acid and did not migrate anodally when electrophoresed at pH 4.5. When the vitamin B_{12}-binding capacity of these plasma was saturated with Co^{60} vitamin B_{12}, no evidence for an unsaturated, anodally migrating $B_{12}BP$ was found.

Chicken plasma contained a protein capable of binding huge amounts of added Co^{60} vitamin B_{12}. The protein was soluble in sulfosalicylic acid and had an electrophoretic mobility at pH 4.5 similar, but not identical to, the human $B_{12}BP$.

Virtually all the vitamin B_{12} of human plasma is bound to a specific protein of plasma.[1, 2] This protein is a constituent of the seromucoid and has a characteristic anodal mobility when it is electrophoresed on a starch block at pH 4.5[2] Vitamin B_{12} is also bound by intrinsic factor,[3, 4] and it has been shown that the bound, as compared to the unbound, vitamin is preferentially absorbed by the small intestine.[5, 6] It is not known whether the entry of vitamin B_{12} into body cells is facilitated or hindered by its binding to a plasma protein.

In this study the electrophoretic behavior of the plasma vitamin B_{12}-binding protein ($B_{12}BP$) of a number of animal species was investigated. It was hoped to find an experimental animal with a plasma $B_{12}BP$ similar to man so that the mechanism of transfer of vitamin B_{12} from plasma to cells could be studied in vitro.

Materials and methods

Human, Rhesus monkey, guinea pig, rat, and chicken venous blood was obtained using heparin as anticoagulant. Rats and guinea pigs were anesthetized with ether, while no anes-

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Received for publication Jan. 16, 1961.

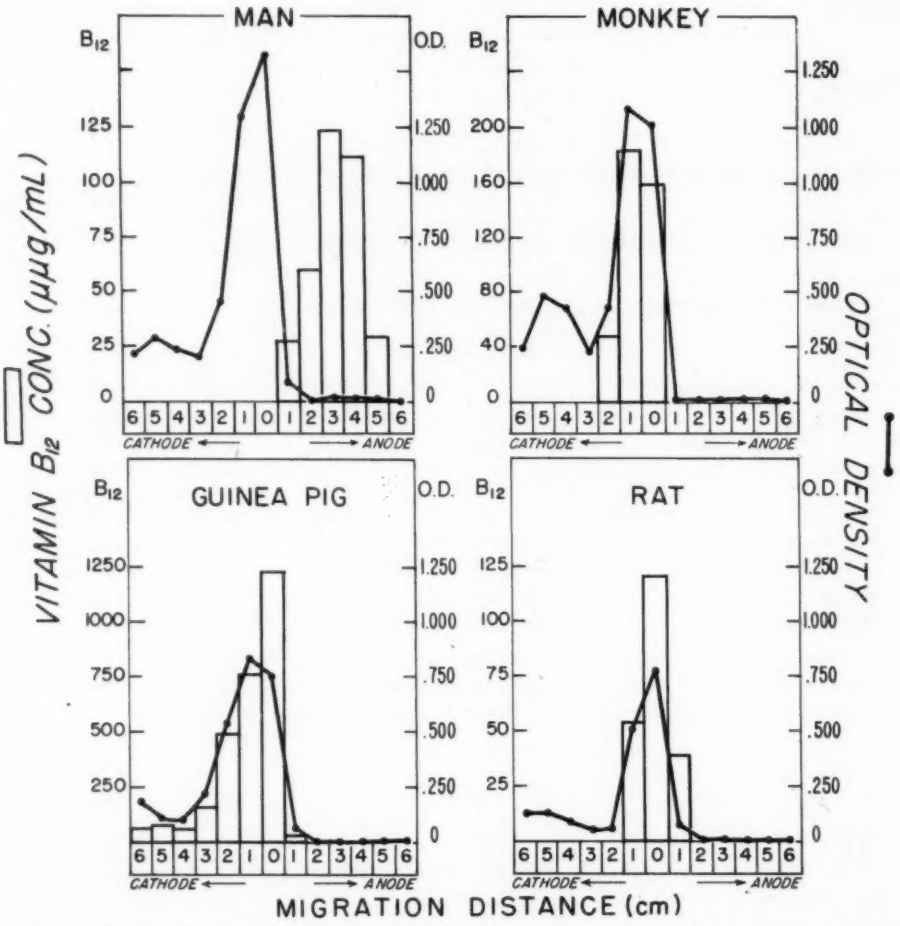

Fig. 1. The vitamin B_{12} concentration of protein fractions of human, monkey, guinea pig, and rat plasma separated by starch block electrophoresis at pH 4.5. Vitamin B_{12} concentration is plotted on the left ordinate and is represented by the open boxes. Note the difference in scale of vitamin B_{12} concentration plotted on the left ordinate for each plasma. Protein concentration is plotted on the right ordinate and is represented by •——•.

thesia was employed with the other animals. The blood from the rats and chickens was pooled in groups of 2. The blood was centrifuged at 4° C. and the plasma was removed. The seromucoid fraction of plasma was prepared by the method of Winzler.[7] One volume of 0.4 M sulfosalicylic acid was added to 1 volume of plasma; the contents were mixed thoroughly, and then centrifuged. The supernatant, containing the seromucoid, was removed and neutralized with NaOH. One or 2 ml. of plasma and neutralized seromucoid from an equivalent amount of plasma each was placed into Visking dialysis bags and dialyzed against 800 ml. of 0.15 M NaCl at 4 to 6° C. for 72 hours, with the NaCl changed every 24 hours. The vitamin B_{12} concentration of the native plasma, the dialyzed plasma, and of the dialyzed seromucoid was determined by *Euglena gracilis* assay.[8] With each analysis, a standard growth curve was constructed from the turbidity given by 1.9, 3.8, 7.6, 15.2, 30.4 and 60.8 μμg of B_{12}, with each level of vitamin B_{12} analyzed in triplicate. In the premilinary phase of the study, the presence of substances which interfered with the growth of *E. gracilis* was found in chicken and rat plasma. The interfering substances of chicken plasma were present uniformly in

high concentration. Thus, when a 1 to 256 dilution of any chicken plasma was added to 30.4 $\mu\mu$g of vitamin B_{12}, no growth of *E. gracilis* was found. Therefore, studies of the chicken plasma B_{12}BP using *E. gracilis* were not possible. The interfering substances in rat plasma were present in much lower concentration. Some rat plasmas were free of interfering substances in plasma dilutions of 1/8 or 1/16. These plasmas only were used in the seromucoid and electrophoresis experiments.

Plasma (1.2 ml.) was electrophoresed at pH 4.5 on starch block using a 0.04 M acetate—0.04 M sodium chloride buffer, as previously described.[2] Following electrophoresis, the block was cut into 1 cm. transverse segments so that the origin was included in one segment (O segment). Other segments were designated as anodal (A) or cathodal (C) and identified by the number of segments from the O segment. Anode and cathode blanks, i.e., 1 cm. areas

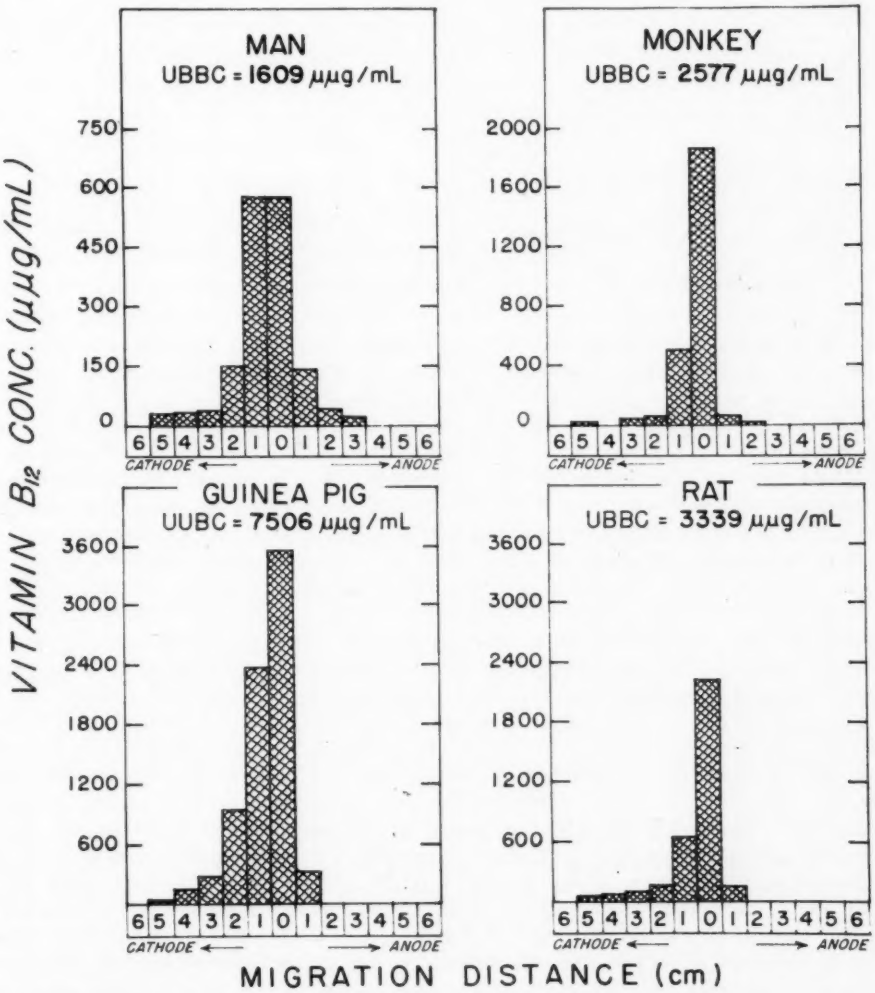

Fig. 2. Distribution of bound (nondialyzable) Co^{60} B_{12} among the protein fractions of human, monkey, guinea pig, and rat plasma separated by starch block electrophoresis at pH 4.5. The concentration of bound Co^{60} B_{12} is plotted on the left ordinate and is represented by the ruled boxes. Note the differences in scale of vitamin B_{12} concentration plotted on the left ordinate for each plasma. The unsaturated vitamin B_{12} binding capacity (*UBBC*) of plasma is also given for each species.

Table I. The vitamin B_{12} concentration of the seromucoid fraction of plasma from 4 mammalian species

Species	*Plasma B_{12} concentration* ($\mu\mu g$/ml.)*	*Seromucoid B_{12} concentration ($\mu\mu g$/ml.)*	*Seromucoid fraction (%)*
Man (4)†			
Mean	353	307	89
Range	253-458	243-438	61-99
Monkey (5)			
Mean	579	58	12
Range	193-1,250	20-96	5-19
Guinea pig (3)			
Mean	2,523	87	3
Range	1,770-3,100	48-126	3-4
Rat (4)			
Mean	225	14	9
Range	138-322	0-35	0-23

*Plasma vitamin B_{12} concentration after dialysis.
†The numbers in parentheses are the number of experiments.

at both ends of the starch block, well beyond the migration path of the plasma proteins, were also cut out. Each segment was suspended in 8 ml. of distilled water and agitated in a Dubnoff shaker at room temperature for 2 hours. The contents were then centrifuged and the supernatant was removed. Protein concentration of each supernatant was determined by a modification[9] of the method of Folin and Ciocalteu.[10] The vitamin B_{12} concentration of each starch segment was determined by *E. gracilis* assay, with the vitamin concentration measured on 3 to 4 dilutions (1/8 to 1/128) of each starch segment. The supernatants from segments C-1, O, and A-1 of rat plasma contained substances which interfered with the growth of *E. gracilis* at dilutions of 1 to 8 but not at 1 to 16. The B_{12} concentration of these segments was determined from the 1/16 to 1/128 dilutions. Substances interfering with the growth of *E. gracilis* were not found in any other segments. Anode and cathode blanks (1 to 8 dilution) usually contained small amounts of vitamin B_{12} ranging from 0 to 3.8 $\mu\mu g$. The average B_{12} concentration of the 2 blanks was subtracted from the vitamin B_{12} concentration measured in each starch segment. Due to the poor accuracy in measuring concentrations of vitamin B_{12} below 3.8 $\mu\mu g$ per sample, the presence of small amounts of vitamin B_{12} in the starch blanks, and the possible random contamination of glassware by small amounts of vitamin, only concentrations of vitamin B_{12} greater than 30 $\mu\mu g$ per starch segment were considered significant.

The electrophoretic mobility of the proteins of plasma that bind added vitamin B_{12} was studied. Plasma from patients with chronic myelogenous leukemia was similarly studied. The electrophoretic mobility of the leukemic B_{12}BP at pH 4.5 is similar to that of the normal, but in contrast to the normal, it is capable of binding large amounts of added vitamin.[2] An amount of $Co^{60}B_{12}$, specific activity 1 μc per microgram, about 3 to 4 times in excess of the plasma binding capacity, was added to 1 ml. of plasma and the unsaturated vitamin B_{12}-binding capacity (UBBC) of plasma was determined by a dialysis technique.[11] An amount of $Co^{60}B_{12}$ (0.2 to 0.3 ml. volume) equal to the determined binding capacity of the plasma was added to 1 ml. of this plasma. The plasma $Co^{60}B_{12}$ mixture was electrophoresed on a starch block at pH 4.5, as described above. The amount of radioactivity in each 1 cm. of the starch block was determined in a plastic well counter.[12] A-1, O, and C-1 to C-3 segments were counted long enough to give a 5 per cent counting error. The A-2 to A-6 segments of all plasma (except from chickens or from patients with chronic myelogenous leukemia) and C-4 to C-6 segments contained negligible amounts of radioactivity and were counted for 8

to 10 minute periods with a 10 minute background count interspersed between each sample count. The average background counting rates during such periods ranged from 392 to 414. Two standard deviations from the average background count was equal to 14 to 16 counts per minute; this amount of radioactivity was equal to 21 to 24 $\mu\mu$g of $Co^{60}B_{12}$. The radioactivity in these segments was considered significant only if its average counting rate was greater than 2 S. D. from the average background count.

Results

Vitamin B_{12} concentration of the seromucoid. The vitamin B_{12} of the plasma of the mammalian species listed in Table I was mostly in the nondialyzable state. Thus, the plasma vitamin B_{12} that was nondialyzable averaged 85 per cent in man, 98 per cent in monkey, 99 per cent in rat, and 88 per cent in guinea pig. The vitamin B_{12} concentration of the seromucoid of human plasma was equal to 61 to 99 per cent of the nondialyzable plasma vitamin B_{12} (Table I). In contrast, rat, monkey, and guinea pig seromucoid contained only a small fraction of the nondialyzable plasma vitamin B_{12}.

Vitamin B_{12} concentration of fractions of animal plasma separated by electrophoresis. Plasma from man, monkey, rat, and guinea pig was electrophoresed at pH 4.5 and the distribution of B_{12} determined (representative sample for each species is shown in Fig. 1). The protein concentration was greatest at either the origin or the C-1 segments in all species. Protein was also distributed among all cathodal segments while the anodal segments contained little to no measurable protein. In man, the B_{12} was virtually all recovered from anodal segments A-2 to A-5, with a peak concentration found at A-3. In contrast, no vitamin B_{12} could be found in these segments in all other species. Almost all of the vitamin B_{12} in these species was found at A-1, O, C-1, and C-2 segments. In some guinea pig plasma, small amounts of vitamin B_{12} were also found in C-4 to C-6 segments. The average recovery of vitamin B_{12} from the starch block was equal to 73 per cent for 5 human, 70 per cent for 5 monkey, 74 per cent for 5 guinea pig, and 52 per cent for 4 rat plasmas.

The distribution of the protein-bound (nondialyzable) Co^{60} B_{12} of mammalian and chicken plasma which had been electrophoresed at pH 4.5 is shown in Figs. 2 and 3. The distribution of protein in all species was generally similar to that in Fig. 1 and is not given in Figs. 2 and 3. The A-2 and A-5 segments of normal human plasma, those segments where the native vitamin B_{12} was found, bound very small amounts of the radiovitamin, representing only 5 per cent of the total plasma-bound radiovitamin (Fig. 2). The bound radiovitamin was concentrated in the O and C-1 segments. In all other animals except the chicken, plasma binding of radiovitamin resembled the pattern found in man, concentrating in the origin and C-1 and C-2 segments. A-2 to A-5 segments contained no Co^{60} B_{12} except a small amount in the A-2 segment of monkey plasma. The unsaturated vitamin B_{12} binding capacity (UBBC) of these plasma is also given in Figs. 2 and 3.

Chicken plasma was capable of binding huge amounts of added Co^{60} B_{12} as compared to the other species (UBBC 180 to 200 mμg per milliliter for 4 such plasma). The binding protein for the added Co^{60} B_{12} was found in the seromucoid fraction of chicken plasma. In contrast to the mammals, almost all Co^{60}

B_{12}, bound by chicken plasma, was recovered from anodal segments with peak concentrations found in A-3, A-2, and A-4 segments, with only negligible amounts found at origin and cathodal segments. The distribution of bound Co^{60} B_{12} in plasma from a patient with chronic myelogenous leukemia, a disease where the ability of the acidic plasma $B_{12}BP$ to bind added B_{12} is greatly increased,[2] is also shown in Fig. 3. Greatly increased binding of Co^{60} B_{12} by the A-2 to A-5 segments was found as compared to the normal human plasma with peak concentrations at A-4, A-5, and A-3 segments. In similar comparative studies with other chronic myelogenous leukemic and chicken plasma, the peak Co^{60} B_{12} concentration of myelogenous leukemic plasma was always found 1 cm. more anodal than the peak Co^{60} B_{12} concentration of chicken plasma.

Discussion

The human plasma $B_{12}BP$ was soluble in sulfosalicylic acid but the $B_{12}BP$ of the monkey, guinea pig, and rat was only minimally soluble in this acid. The human plasma $B_{12}BP$ migrated anodally when electrophoresed on a starch block at pH 4.5, whereas the $B_{12}BP$ of the other 3 mammalian plasma did not move anodally. It should be noted that quantitative recovery of vitamin B_{12} from the starch block was not obtained. This was most marked with rat plasma where only one-half of the vitamin was recovered. The incomplete recovery of vitamin B_{12} from the starch block may be due to (1) incomplete elution of the vitamin from the starch block, (2) difficulty in the measurement of small amounts of the vitamin, and (3) the presence, in rat plasma, of substances interfering with the growth of *Euglena gracilis*. As in a previous study,[2] the anodally migrating $B_{12}BP$ of normal human plasma was virtually saturated, as it could bind only small amounts of added Co^{60} B_{12}. When the vitamin B_{12}-binding capacity of the other mammalian plasma was saturated, no anodally migrating unsaturated $B_{12}BP$ was found. Thus, the anodally migrating type of $B_{12}BP$, as found in human plasma, was not found in the plasma of the Rhesus monkey, guinea pig, and rat. However, its presence in these plasmas in low concentrations cannot be excluded in view of the insensitivity of the methods employed.

Chicken plasma, the only nonmammalian species studied, contained a protein that could bind huge amounts of added vitamin B_{12}, confirming the observations of Gregory and Holdsworth.[13] The chicken plasma $B_{12}BP$ resembles that of the human as (1) it is soluble in sulfosalicylic acid, (2) it behaves as an acidic protein when electrophoresed at pH 4.5, and (3) it has a similar electrophoretic mobility at pH 8.6.[1, 13] However, it is not identical to the human $B_{12}BP$, as its electrophoretic behavior at pH 4.5 was similar but not identical to that of the human $B_{12}BP$. We were unable to study the electrophoretic behavior of the protein binding the in vivo vitamin B_{12} because of the presence in chicken plasma of substances interfering with the growth of *E. gracilis*. From studies of Gregory and Holdsworth[13] with a highly purified specimen of the $B_{12}BP$ of chicken plasma, it seems likely that the protein binding the large amounts of vitamin B_{12} in vitro also contains the in vivo vitamin B_{12}.

A number of interesting questions relating to the evolution of the plasma $B_{12}BP$ in vertebrate species arise from the present findings. These questions may

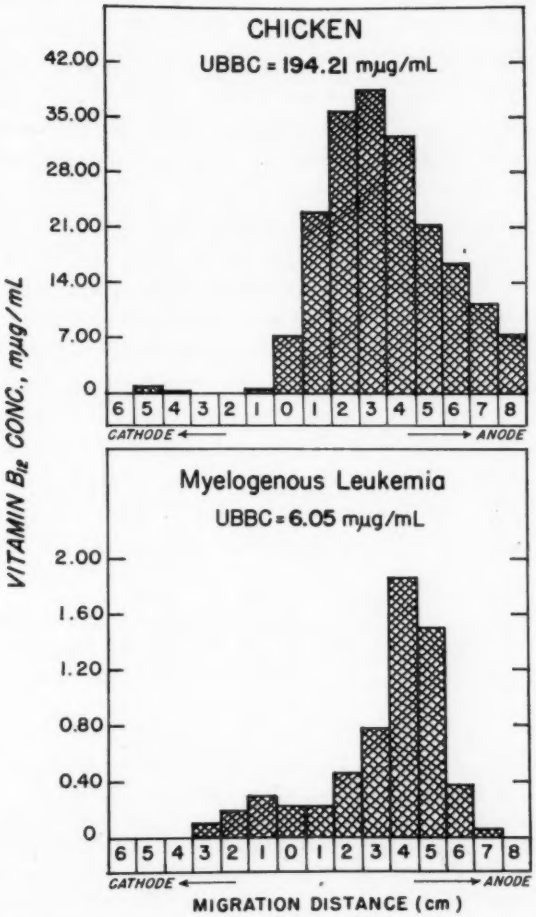

Fig. 3. Distribution of bound (nondialyzable) Co^{60} B_{12} among the protein fractions of human chronic myelogenous leukemic and chicken plasma separated by starch block electrophoresis at pH 4.5. Plasma from a chicken and from a patient with chronic myelogenous leukemia containing bound Co^{60} B_{12} were electrophoresed on the same starch block. The nondialyzable vitamin Co^{60} B_{12} concentration (ruled boxes) is plotted on the left ordinate as millimicrograms per milliliter, in contrast to the micromicrograms per milliliter employed in Figs. 1 and 2. Furthermore, note the difference in the scale of vitamin B_{12} concentration for the 2 plasmas. The unsaturated vitamin B_{12} binding capacity *(UBBC)* of each plasma is also given above.

be answered by a study of the plasma B_{12}BP of lower vertebrate and higher anthropoid species.

Some of the known properties of the human plasma B_{12}BP suggest certain aspects of its chemical structure. It is soluble in sulfosalicylic acid and relatively heat-resistant,[14] properties that have been ascribed to the high concentration of carbohydrate in proteins of the seromucoid.[15] In this regard, chicken plasma B_{12}BP, which is also soluble in sulfosalicylic acid, has been found to contain large amounts of carbohydrate.[13] When serum is electrophoresed at pH 4.5, the human B_{12}BP has an anodal mobility[2] similar to the acidic alpha-$_1$ glycoprotein,

the chief constituent of the seromucoid. The latter protein has an isoelectric point of 2.7 in phosphate buffer[16] and contains 15 to 17 molecules of sialic acid.[17, 18] Popenoe[18] has shown that the sialic acid molecules occur as terminal end groups and are responsible for the acidic isoelectric point of the acidic alpha-$_1$ glycoprotein. The possibility that the acidic isoelectric point of the human and chicken $B_{12}BP$ may be due to terminally located sialic acid molecules is currently under investigation.

The purpose of this investigation was to find an experimental animal with a plasma $B_{12}BP$ similar to man so that the cellular uptake of vitamin B_{12} from plasma to cell could be investigated in vitro. None of the mammals studied fulfilled this criterion. Whether the difference in $B_{12}BP$ between man and mammal signifies a difference in the mechanism of transfer of the vitamin into cells cannot be answered as it is not known whether the protein binding of vitamin B_{12} in plasma facilitates or hinders its entry into the cell. Furthermore, nothing is known of the binding avidities of the various binding proteins. The chicken had a plasma $B_{12}BP$ similar, but not identical, to that found in man. Because of the great biologic difference between the 2 species, further study is needed to ascertain if the mechanism of cellular entry of vitamin B_{12} in man and chicken is similar.

We are indebted to Dr. E. Knobil, who kindly supplied the monkey blood, and to Dr. N. S. Ritter of Merck and Co., Rahway, N. J., who kindly supplied us with Co^{60}-labeled vitamin B_{12}.

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Laboratory methods

A simple technique for prolonging the storage life of I^{131}-labeled proteins and polymers

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A technique permitting greatly increased shelf life of iodinated proteins and polymers is described. Storage of labeled compounds in proximity to a cellophane enclosed anion exchange resin results in continuous removal of dissociated I^{131}. Less than 1 per cent free iodine was present in iodinated albumin, gamma globulin, and polyvinlpyrrolidone during storage for periods of time limited only by the half-life of the isotope.

Continuous in vitro liberation of free iodine from radioiodinated proteins imposes serious limitations upon their usefulness as labels. After a period of 2 to 3 weeks compounds such as radioiodinated serum albumin and gamma globulin generally are considered unsatisfactory for most isotopic procedures due to the eventual contamination of the original material with free I^{131}.[1]

A method has been devised whereby liberated free iodine may be continuously removed and sequestered from I^{131}-labeled protein or polymer solutions by storage of the labeled compounds in the presence of cellulose dialysis bags containing an anion exchange resin. The resin-containing bag is immersed in the labeled protein solutions. Trapping of liberated free I^{131} within the bag not only results in increased purity of the labeled compound during initial use but also significantly increases its shelf life. These advantages prevail regardless of the rate of deiodination or of the initial specific activity of the labeled compound.

Materials and methods

I. Construction of the resin bag. Amberlite IRA-401,* a relatively coarse anion exchange resin, was washed thoroughly with pyrogen-free saline until the supernatant solution was clear.

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Received for publication Feb. 20, 1961.
*Rohm & Haas Co., Philadelphia, Pa.

A section of narrow cellulose dialysis tubing was then tied at one end and loosely filled with approximately one part of the washed resin and one part normal saline. The air was expelled, the opposite end of the tubing tied securely, and the exterior carefully rinsed with saline to insure that no resin adhered to the outer surface of the bag.

The resin bag was then placed in a suitable bottle with a quantity of normal saline sufficient to cover the tubing, after which the bottle was sealed with a rubber cap and autoclaved. The size of the bag and its container were dependent upon the amount of material to be stored. If the bag occupied at least one-sixth of the volume of the solution, satisfactory iodide adsorption could be accomplished without continuous shaking. The method was used to store 10 to 250 ml.

Following sterilization, the saline surrounding the bag was removed and the I^{131}-labeled compound was introduced. Subsequently, samples of the labeled solution were obtained with care being taken not to puncture the resin bag. Although the fluid volume of the 2 compartments in the bottle may vary slightly, a correction in the concentration of the labeled material was considered unnecessary.

II. Determination of free I^{131} in stored proteins and polymers. The trichloroacetic acid (TCA) soluble (nonprotein-bound) activities of serum albumin-I^{131} and gamma globulin-I^{131} solutions were determined by diluting the proteins in 2 ml. of 5 per cent albumin before the addition of 4 ml. of cold 10 per cent TCA. The samples were then refrigerated for 45 minutes before they were centrifuged and washed twice with cold TCA. The TCA-soluble radioactivity was assumed to represent free iodine. Free iodine in labeled polyvinylpyrrolidone (PVP) preparations was determined by passage through an ion exchange resin column (P-B-I Rezikit).*

III. Evaluation of the resin bag for efficiency of iodide removal during the first 24 hours. It was found that iodide removal often was necessary prior to initial use of a labeled compound. In order to determine whether the system reported herein would effectively remove the contaminating free iodine during the first 24 hours of contact the following experiment was performed.

A tracer quantity of NaI^{131} in saline was introduced into a bottle containing a resin bag and the bottle was refrigerated for 24 hours. At the end of this time the bag was removed, rinsed, and the percentage of total I^{131} radioactivity which had been adsorbed by the resin was determined. It was found that 90 per cent of the initial radioactivity had been trapped in the resin compartment.

In order to determine if shaking would significantly alter the I^{131} adsorption by the resin an identical bottle was shaken gently at room temperature for 24 hours. Ninety-five per cent of the I^{131} activity was then found within the bag.

IV. Evaluation of the resin bag for prolonged storage. Since serum albumin-I^{131} is probably the most commonly employed radioiodinated material in use, one lot of RISA† and one of RISA-M* were stored in the described manner to demonstrate the maintained stability after prolonged storage.

Upon arrival, the 2 lots of iodinated albumin were diluted with saline and one-half of each solution was transferred to a resin bottle. The remainder was left in the original shipping bottle and all samples were then stored in the refrigerator for 2 months.

The integrity of the stored RISA and RISA-M was evaluated by performing the following procedures: (1) After 4 and 8 weeks of storage a TCA precipitation was performed in duplicate on aliquots of all samples, (2) after 8 weeks a paper radioelectrophoretic pattern‡ was obtained on all samples, and (3) after 10 weeks a plasma disappearance rate was determined on the 2 lots which had been stored in the presence of the resin bag.

The results of the TCA precipitations are shown in Table I. As can be seen in the table, the nonprecipitable fraction was reduced from 3.6 to 0.5 per cent in the lot of RISA and from 3.2 to 0.3 per cent in the lot of RISA-M following 8 weeks of storage in the presence of the resin bags. The radioelectrophoretic patterns of the stored material showed no indication of

*E. R. Squibb & Sons, New York, N. Y.
†Abbott Laboratories, North Chicago, Ill.
‡Procedure B, Spinco Model R. Paper electrophoresis system.

Table I. Per cent of free iodine in 2 lots of iodinated human serum albumin stored for 8 weeks with and without the resin bag

	TCA soluble fraction in RISA		*TCA soluble fraction in RISA-M*	
Weeks following assay date	*Portion left in shipping bottle (%)*	*Portion transferred to bottle containing resin bag (%)*	*Portion left in shipping bottle (%)*	*Portion transferred to bottle containing resin bag (%)*
4	3.46	0.50	2.46	0.33
4	3.21	0.55	2.47	0.34
8	3.63	0.49	3.16	0.37
8	3.60	0.49	3.29	0.15

altered or degraded albumin-I^{131}. The plasma disappearance rates performed on the 2 albumin preparations, in spite of 10 weeks of storage, showed no alteration in the expected "die-away curve"[2, 3]; after 45 minutes 92 per cent of the RISA and 94 per cent of the RISA-M were present in the patients' plasma.

Discussion

The technique described above for removing free I^{131} from stored iodinated proteins and polymers serves a twofold purpose. First, it increases the initial accuracy of the procedures to be performed by removing free I^{131} present at the time of arrival of a shipment. Second, it provides a means of storing labeled compounds for longer periods of time than was possible previously. Much saving in time and materials is accomplished when I^{131} compounds may be maintained sterile and devoid of free iodide for as long a time as physical decay of the isotope permits.

Since the iodinated compounds employed in this study were intended for intravenous use in human beings, no attempt was made to determine the feasibility of direct addition of the resin to the compounds, thereby eliminating the dialysis bag. If, however, the stored compounds are intended for in vitro use only, it is possible that the cellulose bag may be unnecessary, provided that direct contact with the resin does not result in alteration of the labeled compound or in its adsorption to the resin.

This procedure has been satisfactorily employed in this laboratory for the past 16 months. Radioiodinated albumin, gamma globulin, and PVP were stored in this manner. Before the availability of this storage procedure it had been necessary either to obtain new preparations or to redialyze and resterilize old preparations almost weekly. Following the institution of this method, most of the labeled preparations were used for 4 to 5 weeks without further inconvenience. At no time during use was the free I^{131} in excess of 1 per cent. Preparation of the 2 compartment system is simple and inexpensive and resin bottles prepared in advance are thus available at all times.

The maximum storage period of labeled solutions depends upon the required specific activity, and, theoretically, upon the possibility of the formation of nondialyzable self-degraded or altered products of some iodinated compounds.[4]

Degradation products which are dialyzable could in all probability be removed by the addition of an appropriate resin to the bag.

The method described here may lend itself to purification of other isotopically labeled nondialyzable compounds, and possibly of unlabeled, unstable compounds. Compounds of low molecular weight which are dialyzable could possibly be stored in this manner if a suitable resin were found which bound only the undesirable component while permitting free flow of the compound through the membrane.

We wish to express our sincere appreciation to Dr. Seymour Eisenberg for his interest and assistance in this study, and to Drs. J. R. Rubini, Ben Friedman, and R. H. Unger for valuable assistance in the preparation of the manuscript.

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A modified technique for periodate inactivation of serum mucoproteins

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A modified technique for periodate treatment of serum to inactivate mucoprotein inhibitor of myxovirus hemagglutination is described. Periodate salts, normally only slightly soluble at physiologic pH, are solubilized in higher concentration in 0.1 N potassium hydroxide and the solution is neutralized with concentrated hydrochloric acid. This refinement will allow initial serum dilutions of 1:2 for hemagglutination-inhibition tests rather than requiring the 1:5 to 1:7 dilution usually employed, and will permit serologic diagnosis of myxovirus infections even though the convalescent serum hemagglutination-inhibition titers are as low as 1:4.

The use of periodate to destroy nonspecific mucoprotein inhibitor of myxovirus (influenza and related viruses) hemagglutination is well established.[1] Periodate salts, however, are only sparingly soluble in water at physiologic pH values, and in order to obtain enough of these compounds to inactivate all inhibitor it is necessary to employ relatively large volumes of solution in proportion to the volume of serum being treated. Burnet and Lind,[2] using 0.01 M periodate, and the Expert Committee on Respiratory Virus Diseases,[3] recommending 1/90 M periodate, described procedures leading to serum dilutions of 1:5 to 1:7, respectively. Such high initial serum dilutions limit the usefulness of this procedure in routine treatment of serum, for low levels of antibody may not be detected[4] and diagnostic (fourfold) convalescent increases in hemagglutination-inhibition antibody may be overlooked. The solubility of periodate salts may be increased by dissolution in alkali.

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Supported by United States Public Health Service Grant No. E-1131.
Received for publication Dec. 27, 1960.
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Materials and methods

Three hundredths molar (0.03 M) potassium metaperiodate was prepared in 0.1 N potassium hydroxide by shaking it at room temperature for 30 minutes; the solution was then brought to pH 7.6 by dropwise addition of concentrated hydrochloric acid. Eight-tenths volume (0.8 vol.) of the freshly made, neutralized solution was added to 1 vol. of serum and allowed to react for 1 hour at room temperature; the excess periodate was then reduced with 0.2 vol. of 12 per cent glycerol-saline. This procedure yields a serum dilution of 1:2.

Results

Experiments with several human sera (Table I), using the very inhibitor-sensitive egg-ferret–mouse-egg strain of Asian influenza virus as antigen in a hemagglutination-inhibition test, showed 0.03 M periodate, 1/90 M periodate by the method of the Expert Committee[3] or the receptor-destroying enzyme of *Vibrio comma* to be equally effective in destroying nonspecific inhibitor without altering the titer of specific antibody.

Particularly illustrative of the diagnostic value contained in low serum dilutions are Cases Nos. 66 and 987 (Table I). Using an initial serum dilution of 1:7, neither of these patients could be serologically diagnosed as afflicted with Asian influenza, for the convalescent increment was equivocal in Case No. 66, and no antibody was detected in either the acute or convalescent sera of Case No. 987. When the serum dilution was lowered to 1:2, a clear fourfold con-

Table I. Effect of different methods of inhibitor destruction on the hemagglutination-inhibition titer* of human sera against Asian influenza virus

		Method of inhibitor destruction			
Case No.	*Human serum*	*None*†	*RDE*‡	*0.03 M KIO_4*	*1/90 M KIO_4*
66	Acute	nd§	< 4	< 2	< 7
	Convalescent	nd	16	8	7
663	Acute	1,024	32	16	28
	Convalescent	1,024	512	512	448
686	Acute	512	8	4	7
	Convalescent	512	32	16	28
904	Acute	512	4	8	7
	Convalescent	512	32	32	28
909	Acute	nd	< 4	2	< 7
	Convalescent	nd	8	16	14
912	Acute	256	8	16	7
	Convalescent	256	16	32	14
987	Acute	nd	nd	< 2	< 7
	Convalescent	nd	nd	4	< 7
1,181	Acute	nd	4	2	< 7
	Convalescent	nd	4	2	< 7

*Reciprocal titers are shown; 0.25 per cent human type O red blood cells settled at 4° C.
†Serum was heated at 56° C. for 30 minutes.
‡Receptor destroying enzyme of *Vibrio comma*.
§Not done.

valescent rise was noted in both instances. Asian influenza virus was isolated from both these persons.

Usually, human type O erythrocytes were used. Experiments with rooster and guinea pig red blood cells indicated that serum treatment with 0.03 M periodate did not result in increased serum agglutination of the rooster cells, but may have increased the tendency of serum to agglutinate guinea pig cells.

It seems possible that the foregoing refinement of the conventional periodate method for serum treatment may prove suitable for routine use in surveys assaying antibody levels and rates of infection with myxoviruses in virgin populations.

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Current methods for determining the volume of a Warburg manometer and flask

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Two currently recommended methods for determining the volumes of Warburg manometers and flasks have been compared with the original procedures from which they were derived as to precision, validity, and the effect of position of the apparatus on volume determinations. Analyses of variance showed that good replication occurred in all 68 tests, with 5 replicates per test. A statistically significant difference was found to exist between the calculated manometer volumes when the manometer and micrometer calibrator were held in the horizontal, vertical, or inverted positions. This positional effect was obscured when the relatively small manometer volumes were combined with the much larger flask volumes. A statistically significant difference exists between the total manometer and flask volumes as calculated by the one-step and the standard Hg calibration methods. A comparison of these data with the original data of Lazarow indicates a need for review and revision of current textbook recommendations. Either the Lazarow one-step water or the standard Hg method, each with a precision of about 1.0 per cent, should be selected for the calibration of Warburg manometric glassware.

Many methods exist for determining the volume of a Warburg manometer and flask, e.g., 12 methods are cited by Umbreit and coauthors.[1] Basically, however, all methods can be classed into either of two types: one in which the manometer and flask volumes are determined separately (the "standard" method) or one in which a single volume determination of manometer plus flask is made (the "one-step" method). The two methods of Lazarow are probably the most representative of the two classes. The standard method, in which the flask volume is determined by the weight and density of Hg that completely fills the flask and the manometer volume determined directly with the Lazarow water-filled micrometer calibrator,[2] and the one-step method, in which the combined manometer and flask volume is read directly from the modified water-filled micrometer calibrator of Lazarow.[3]

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Supported in part by United States Public Health Service Grant No. B-1541.
Received for publication Feb. 27, 1961.

Table I. Volume (ml.) of Warburg manometer and flask

Unit	*Flask volume*	*Mean ± standard error of mean*			
		*Horizontal**	*Vertical**	*Inverted**	*One-step*
1	18.348	0.5499 ± 0.0052	0.5353 ± 0.0024	0.5615 ± 0.0032	18.58 ± 0.20
2	20.584	0.3914 ± 0.0051	0.3568 ± 0.0121	0.3195 ± 0.0043	20.64 ± 0.28
3	19.087	0.4507 ± 0.0020	0.4262 ± 0.0017	0.4739 ± 0.0046	17.94 ± 0.28
4	16.264	0.4263 ± 0.0031	0.4061 ± 0.0026	0.4437 ± 0.0043	17.26 ± 0.14
5	17.992	0.4164 ± 0.0020	0.4000 ± 0.0018	0.4286 ± 0.0010	18.22 ± 0.17
6	17.787	0.4396 ± 0.0021	0.4091 ± 0.0014	0.4442 ± 0.0029	18.30 ± 0.10
7	17.180	0.3790 ± 0.0011	0.3690 ± 0.0032	0.3925 ± 0.0011	18.06 ± 0.14
8	17.081	0.3838 ± 0.0024	0.3577 ± 0.0021	0.3978 ± 0.0019	17.44 ± 0.20
9	17.702	0.4329 ± 0.0027	0.4149 ± 0.0019	0.4468 ± 0.0032	18.02 ± 0.16
10	16.351	0.4255 ± 0.0017	0.4048 ± 0.0026	0.4407 ± 0.0021	16.67 ± 0.02
11	20.690	0.4679 ± 0.0014	0.4510 ± 0.0011	0.4861 ± 0.0031	20.32 ± 0.10
12	16.643	0.4126 ± 0.0038	0.3950 ± 0.0042	0.4284 ± 0.0053	17.46 ± 0.12
13	17.930	0.4167 ± 0.0023	0.3967 ± 0.0030	0.4334 ± 0.0018	17.74 ± 0.27
14	16.427	0.4072 ± 0.0027	0.3913 ± 0.0019	0.4219 ± 0.0028	16.64 ± 0.07
15	17.242	0.4310 ± 0.0013	0.4045 ± 0.0015	0.4531 ± 0.0023	17.54 ± 0.07
16	16.971	0.4373 ± 0.0016	0.4197 ± 0.0016	0.4551 ± 0.0032	17.82 ± 0.05
17	21.569	0.3700 ± 0.0020	0.3492 ± 0.0018	0.3927 ± 0.0015	22.16 ± 0.20

*Manometer volume only.

Within each class modifications have been designed to increase precision and speed of calibration. Modifications of the calibration methods of Lazarow, utilizing Hg instead of water as the calibration fluid, have gained in acceptance in recent years, as evidenced by that fact that they are recommended in current textbooks.[1, 4] The use of Hg circumvents the problem of the wetting of glass surfaces, thus permitting unlimited replications to be made; however, a new variable is also introduced—the hydrostatic pressure of the column of Hg within the calibrator and the manometer. Data have been obtained with the Hg calibration procedures which permit comparisons of precision and validity to be made with the original water calibration data of Lazarow.[2, 3] Statistical analyses were performed using the methods of Mather.[5]

Results and discussion

The results of 340 determinations of manometer and flask volumes are summarized in Table I. Each value represents the mean of 5 replicates using a mercury-filled micrometer calibrator.

An analysis of variance of the standard data in Table I indicates that there is a statistically significant difference ($p < 0.001$) between the calculated volumes for a single manometer when the mercury-filled micrometer calibrator and manometer are alternately held in the horizontal, vertical, and inverted positions, i.e., the well of the micrometer calibrator opens horizontally, upward, and downward, respectively. The manometer volumes were found to be related to the position of the manometer and calibrator in the earth's gravitational field, i.e., the greater the hydrostatic pressure of the Hg column (inverted position) the greater the calculated volume.

In the determination of the manometer volume by the standard Hg method, it is essential that the portion of the manometer measured be completely filled with mercury. Unfortunately, even though a very exacting technique is used, a few tiny air pockets are always trapped in the system, especially near the stopcock assembly. The effect of gravity on the Hg column, with its high hydrostatic pressure, is on the compression and expansion of the trapped air pockets.

When relatively small manometer volumes (range: 0.3117 to 0.5690 ml.) were added to the much larger flask volumes (range: 16.624 to 21.569 ml.) the statistically significant effects of position on the small manometer volume determinations were obscured, i.e., there was no statistically significant effect of positions ($p > 0.5$) on total volumes of flask and manometer. This result occurs when 2 figures of vastly different magnitudes are summed. The precision of the weighing of the Hg for the determination of the flask volume by the standard method was performed with an accuracy of 0.05 per cent. The variance of the larger figure (the flask) is the major determinant of the variance and consequently of the statistical analyses of the resulting sum.

With the one-step Hg method the volume being measured, i.e., manometer plus flask, is completely filled with gas during calibration. Consequently, the hydrostatic effect of the column of Hg will be much greater on the one-step Hg method with the relatively large gas phase than on the standard mercury method with the tiny air pockets, i.e., there is a statistically significant difference, $p < 0.001$, between the total volumes as determined by the standard and the one-step Hg methods.

Two of the most important qualities of any calibration procedure are precision and validity; precision in that the replicate values afford the best possible estimate, i.e., the least possible error, of the mean value and validity in that the precise mean value is the best estimate of the "true" mean. Due to the wetting of the glass surfaces with water on the initial determination of each manometer volume by the standard water method, Lazarow discounted the first readings and calculated precision on the basis of the second and third wet replicates. Precision of replication of wet manometer volumes by the Lazarow water method was 0.34 per cent, based on data from two units.[2] The same data showed that a mean per cent error correction factor of 2.42 per cent should be included in each volume determination. This correction factor compensates for the validity error introduced by using but one dry manometer volume determination as the basis for calculating the volume of the wetting film of water. The corrected mean precision for the 2 units is thus 2.76 per cent for the standard Lazarow water method compared to a precision of 1.00 ± 0.12, 1.08 ± 0.12, and 1.20 ± 0.28 per cent (mean of 1.09 per cent) by the standard Hg method in the horizontal, inverted, and vertical positions, respectively.

Lazarow stated that with the one-step water method the average error, assumed to be of precision, of 9 determinations equaled 1.0 ± 0.7 per cent. A precision of 1.54 ± 0.17 per cent is reported here for the one-step Hg method, which produced a statistically greater per cent error (1.54 with $p < 0.02$) than did the standard mercury method, as repesented by the mean of the 3 positions (1.09 per cent).

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Polyethylene constriction pipettes

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Herein are described the construction, calibration, and accuracy of polyethylene constriction pipettes for volumes ranging from 0.3 to 300 µl. In our laboratory they are preferred to glass constriction pipettes because they are easy to make and are not fragile, because fluids do not adhere to their walls and because, in our hands, they are more accurate at the smallest volumes than most glass constriction pipettes we have tested. However, fluids of temperatures higher than 40° C. will change the shape of polyethylene pipettes.

Glass constriction pipettes[1] (Linderstrom-Lang-Levy or Carlsberg pipettes) have long been used successfully for micro- and ultramicrochemical methods. The mean standard deviation (S.D.) of pipettes made in our laboratory increases rapidly with decreasing pipette volumes.[2] For this reason we developed polyethylene micropipettes, which deliver 0.4 to 10 µl[2] and, having a mean S.D. of ± 0.42 per cent, were more accurate than glass constriction pipettes of the same magnitude. These polyethylene micropipettes consist of polyethylene capillaries mounted in a glass holder. They are made easily for any volume required and are very easy to operate. However, the diameter of the glass holder makes it difficult, if not impossible, to pipette into the narrow micro–test tubes used for ultramicromethods. In order to overcome this difficulty, we have developed polyethylene constriction pipettes, which can be used with ultramicro–test tubes.

Polyethylene pipettes have several advantages: (1) They are unbreakable; (2) since the material cannot be wetted the accuracy of delivery of fluids is increased; (3) they can be made with ease. Polyethylene has a melting point of about 115° C., but its consistency changes between 45° and 100° C. Therefore,

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Supported in part by Grant No. (H-5975) from the National Institutes of Health, Bethesda, Md., and in part by Contract No. DA-49-007-MD-637 from the Surgeon General's Office, U. S. Army.

Received for publication March 13, 1961.

Fig. 1. Pair of cable stripping pliers used for forming the constriction in pipettes < 10 μl. When closed the jaws do not meet but overlap closely. The opening can be adjusted as required by the set screw.

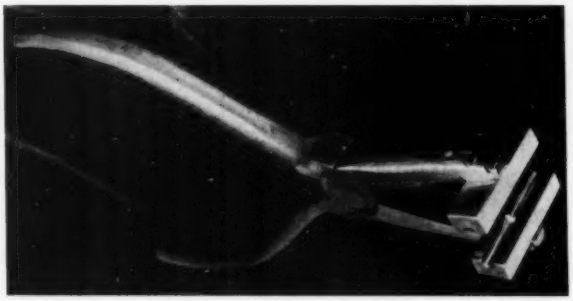

Fig. 2A. Pair of mould pliers for pipettes > 10 μl.

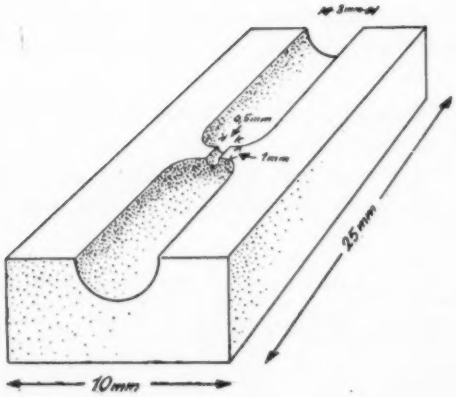

Fig. 2B. Sketch of one of the identical moulding jaws of the pliers shown in Fig. 1*A*.

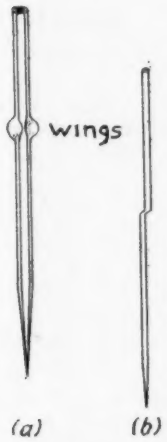

Fig. 3. Polyethylene constriction pipettes: *a*, volumes > 10 µl; *b*, volumes < 10 µl.

after they have been made, the pipettes should never be exposed to temperatures above 40° C., to avoid any change in shape or in the constriction; fluids with temperatures higher than 40° C. should not be pipetted with polyethylene pipettes.

We have used polyethylene constriction pipettes for nearly 2 years, and the original pipettes we made are still in use and have not changed their volume.

Materials and methods

Procedure for making polyethylene constriction pipettes.

PREPARATION OF TUBING. For making polyethylene pipettes, tubing with the adequate diameter has to be selected (Table I).

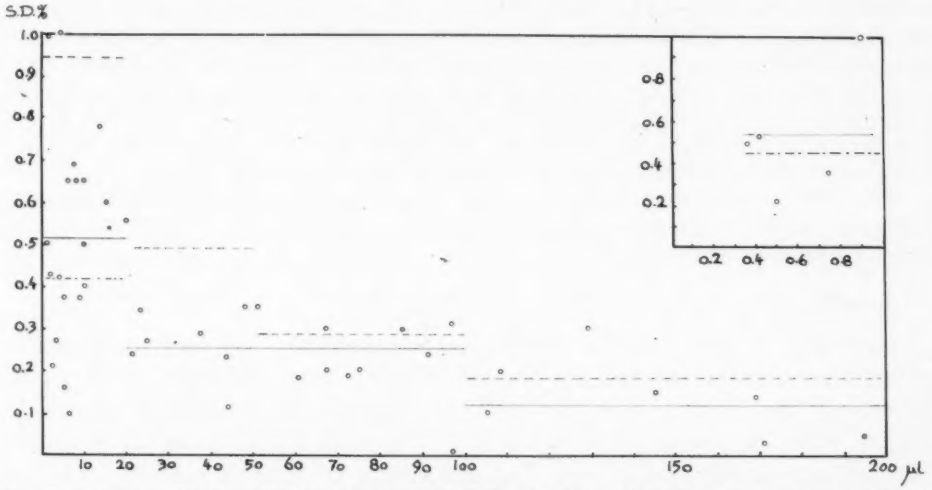

Fig. 4. Accuracy of the pipettes. Percentage S.D. has been plotted against pipette volumes. The points given are for individual polyethylene constriction pipettes. The mean S.D. for each group is indicated by solid lines. For comparison the mean S.D. for glass constriction pipettes (--------) and polyethylene capillary pipettes (- - - - - -) is given. For 2 pipettes (210 and 301 µl) the points are not given but the S.D. (0.05 and 0.03 per cent) has been taken into account.

Table I. Selection of polyethylene tubing

Pipette volume (μl)	*Diameter (mm.)*	
	Outside	*Inside*
< 1	1.0	0.6
1—10	1.3	0.8
10—50	2.8	1.6
> 50	3.4	2.1

The tubing is cut into pieces, approximately 15 cm. long, and left overnight in a detergent solution, rinsed carefully with water, and dried by sucking acetone or alcohol followed by air through the tubing.

PULLING THE TIP. A piece of tubing is heated approximately 3 cm. from one end high over a small flame. It is rotated during the heating. As soon as the material becomes soft, the tubing is pulled carefully at one or both ends and the tension is maintained until the polyethylene is cool. The tip can then be cut to the desired length.

PRESSING THE CONSTRICTION. For pipettes with volumes < 10 μl a simple cable stripper with carefully polished edges is used to press the constriction (Fig. 1). For pipettes with volumes > 10 μl a pair of forming pliers was developed* (Fig. 2). The desired volume of water is sucked into the pipette either from a balance pan or directly from a calibrated pipette. The position at which the constriction will be made is marked with a pencil 1 or 2 mm. above the water meniscus. The pipette is then emptied and dried. The pliers are heated in boiling water, and the constriction is pressed in at the marked point. The pliers are kept closed for about 2 minutes and then are cooled under running tap water before being opened. One point is of critical importance. On each side of the constriction a "wing" is formed, consisting of two laminae (Fig. 3). Unless these have been joined in firm apposition after heating, fluid will be sucked between the laminae. The constriction should be checked under the microscope and the pipettes discarded if the laminae are open. If the constriction has been properly made, the fluid meniscus will be held at the narrowest point of constriction, except when an increase of pressure is applied to overcome the resistance. The pipettes are operated by mouth, using rubber tubing. The ends of very thin pipettes must be inserted in polyethylene tubing with wider bore, to which the rubber tubing can be fitted for suction.

CALIBRATION OF THE PIPETTES. Pipettes with volumes > 10 μl can be calibrated gravimetrically with water; smaller pipettes are calibrated colorimetrically with p-nitrophenol in alkaline solution, as described previously.[2, 3] Corrections for too large a volume can be made by cutting a piece from the tip. Otherwise, the pipettes are used with a correction factor. With practice the desired volume can be attained within ± 2 or 3 per cent.

CLEANING OF THE PIPETTES. After use, the pipettes are rinsed with water. If necessary, they may be soaked in a detergent solution to which 1 per cent Na_3PO_4 is added. The pipettes are dried with ether or alcohol.

Results

Accuracy of the pipettes. In Fig. 4 the standard deviations are plotted against the pipette volumes for the polyethylene pipettes constructed in our laboratory. The pipettes were classified into 4 groups of increasing volume, 0.3 to 1.0 μl (Fig. 4, inset), 1.0 to 20 μl, 20 to 100 μl, and 100 to 300 μl. The mean S.D. for each group (solid lines) decreased with increasing volume, being, respectively, 0.51, 0.51, 0.25, and 0.11 per cent. The dotted lines give the mean S.D. for the glass constriction pipettes made in our laboratory.[2] (We have found that

*The forming pliers may be obtained from Acme Model Works, Inc., 200 North Jefferson Street, Chicago, 6, Ill.

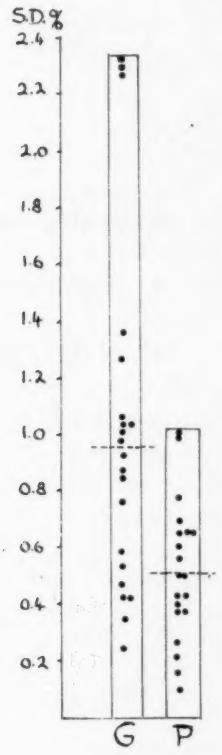

Fig. 5. Distribution of S.D. for glass (*G*) and polyethylene (*P*) constriction pipettes in the range from 1 to 20 μl. The mean S.D. for each group is indicated by (-----).

the S.D. for many commercial glass constriction pipettes is much higher.) In the range up to 50 μl, the mean S.D. for polyethylene constriction pipettes was lower than for glass constriction pipettes. This is especially true for pipettes < 20 μl in volume. But it should be pointed out that glass constriction pipettes with a S.D. lower than ± 0.5 per cent can be made. In Fig. 5 the standard deviations for glass and polyethylene pipettes (group 1— 20 μl) are shown. From the distribution it can be seen that whatever criteria are used in selecting pipettes for accuracy, a higher percentage of the polyethylene pipettes fulfill the requirements. However, the mean standard deviation of the polyethylene constriction pipettes was higher than that for polyethylene capillary pipettes, which was found to be 0.42 per cent (interrupted lines).

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Albumin adsorption in paper electrophoresis: A significant variable in the quantitation of serum protein fractions

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The quantitative aspects of serum albumin adsorption in a frequently used filter paper electrophoretic technique have been investigated by elution studies. It was found that the magnitude of albumin adsorption was not constant, but instead correlated approximately with the absolute quantity of albumin applied. The observations define further limitations of filter paper electrophoresis for quantitation of serum protein fractions.

Early in the development of the technique of serum protein fractionation by paper electrophoresis it was recognized[1] that partial adsorption of serum albumin occurred in the path of migration. This phenomenon of adsorption, or "trailing," produces both an erroneous decrease in the albumin peak and a false increase in the globulin fraction (except the gamma portion). In cases of pathologic sera, in which instances the albumin fraction is frequently reduced, the loss in the "trail" becomes of even greater significance in the appraisal of the resultant patterns. Thus, to quantitate accurately the serum protein fractions separated by paper electrophoresis, the adsorbed trail of albumin must be accounted for in the final calculations.

Previous investigators[2-6] of this phenomenon had interpreted their data to demonstrate that the quantity of albumin adsorbed per unit area was constant for a specific electrophoretic technique irrespective of the absolute amount of albumin applied. These studies utilized both densitometry and elution for quantitation of the albumin-bound dye. More recently, further studies have been

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Supported by the Michigan Chapter of the National Kidney Disease Foundation.
Received for publication Dec. 6, 1960.
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reported[7, 8] which are at variance with the earlier conclusion regarding the fixed quantity of the albumin trail. These later studies were limited to densitometry only.

The frequent clinical usage of serum protein fractionation by paper electrophoresis suggested that further evaluation of trailing by elution studies would be of value. By elution methods, variables attributable to photodensitometry would be avoided. Furthermore, by selection of an electrophoretic technique which had been widely used, the data would have greater general application. In the study to be presented in this communication, a commercially available electrophoretic apparatus* was utilized with strict adherence to the recommended methodology.[9, 10] Elution studies were used throughout.

The observations to be presented confirm the recent densitometric studies[7, 8] and demonstrate that the amount of albumin absorbed in the trail is not of constant magnitude, showing instead a rough correlation to the absolute quantity of albumin applied to the paper. Attempts to prevent adsorption by prior migration of albumin through the filter paper were not successful; furthermore, they showed that saturation of the filter paper by albumin was not attained by the procedures employed. The results presented in this study emphasize the difficulty of quantitation of serum protein patterns separated by paper electrophoresis.

Materials and methods

A. Albumin. Varying quantities of crystallized human serum albumin† were dissolved in 0.90 per cent NaCl with determination of the final albumin concentrations by the phenol reagent method of Greenberg.[11] The purity of the human serum albumin was verified by estimations of its average molecular weight using the method of Adair and Robinson[12] after direct measurements of the oncotic pressure of the albumin in solution. The average molecular weight thus determined was 71,500.

B. Filter paper. Two types of filter paper strips were investigated separately in the study. The first, Schleicher and Schuell 2043-A mgl., is a thin type of paper, while the second, Whatman No. 3, is a fairly thick strip.

C. Electrophoresis. The inverted V-form of electrophoretic cell designed by Williams and associates[10] for paper electrophoresis, which is commercially available, was utilized. Veronal buffer of pH 8.6 and ionic strength 0.075 was used. A commercially available power source for DC current was employed. Repeated electrophoretic separations of varying concentrations of the purified albumin were made. On each run, a single concentration of albumin was applied to all 8 strips of identical paper.

D. Procedure. (1) Samples of 0.006 ml. of the test protein solution were applied to Schleicher and Schuell 2043-A mgl. strips and treated as recommended.[9] (2) Samples of 0.01 ml. of the test protein solution were applied to Whatman No. 3 strips and treated as previously described.[10]

E. Densitometry. Subsequent to bromphenol blue staining, the strips were exposed to NH_4OH vapor in a closed container for ½ hour, and densitometric scanning was performed. A Spinco Model RB Analytrol was utilized for scanning the Schleicher and Schuell strips, and a Spinco Model RA Analytrol for scanning the Whatman No. 3 strips.

*Spinco Model F cell, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.
†Kindly supplied by Dr. F. F. Johnson of Cutter Laboratories, Berkeley, Calif.

Table I. Results of elution studies upon varying concentrations of crystallized human serum albumin separated by electrophoresis upon Schleicher and Schuell 2043-A mgl. paper and Whatman No. 3 paper

No. of pairs	*Column 1. Quantity of albumin applied (μg)*	*Column 2. Amount of dye* in complete strip (μg)*	*Column 3. Amount of dye* in "band" (μg)*	*Column 4. Amount of dye* in "trail" (μg)*	*Column 5. Total dye:* "band" and "trail"† (μg)*	*Column 6. Area of "trail" (cm.²)*	*Column 7. Dye*/cm² "trail" (μg)*	*Column 8. % of total dye lost in "trail"*
A. Schleicher and Schuell 2043-A mgl. paper								
I, II	239	219, 221	212, 203	14, 12	226, 215	14.5, 15.3	0.97, 0.78	6.2, 5.6
III, IV		215, 246	215, 206	15, 12	230, 218	15.9, 14.7	0.94, 0.82	6.5, 5.5
Average	239						0.88	6.0
I, II	189	215, 206	183, 168	15, 14	198, 182	15.5, 16.6	0.97, 0.84	7.6, 7.7
III, IV		125, 210	180, 203	12, 9	192, 212	15.6, 13.9	0.77, 0.65	6.3, 4.2
Average	189						0.81	6.5
I, II	160	132, 123	129, 116	6, 5	135, 122	12.0, 13.2	0.50, 0.45	4.4, 4.9
III, IV		126, 137	108, 123	5, 5	113, 128	14.1, 14.1	0.35, 0.35	4.4, 3.9
Average	160						0.41	4.4
I, II	80	44, 50	41, 39	8, 5	49, 47	19.6, 20.0	0.41, 0.40	16.3, 17.0
III, IV		53	41, 45	5, 5	48, 50	20.4, 18.3	0.25, 0.27	10.4, 10.0
Average	80						0.33	13.4
I, II	52	14, 15	8, 12	6, 5	14, 17	25.0, 23.1	0.24, 0.22	42.9, 29.4
III, IV		15, 17	9, 12	5, 3	14, 15	23.4, 22.3	0.21, 0.13	35.7, 20.0
Average	52						0.20	32.0
B. Whatman No. 3 paper‡								
4	428	199 ± 11	180 ± 3	24 ± 2	204 ± 5	21.8 ± .2	1.10 ± .09	11.8
3	330	178 ± 8	146 ± 10	18 ± 3	164 ± 13	21.6 ± .6	0.83 ± .12	10.9
4	266	134 ± 8	108 ± 6	17 ± 3	126 ± 5	23.8 ± 1.0	0.75 ± .13	14.1
4	162	86 ± 5	64 ± 4	15 ± 3	79 ± 2	16.9 ± 3.0	0.87 ± .12	18.6
4	87	26 ± 9	9 ± 4	12 ± 2	21 ± 3	19.9 ± 1.4	0.62 ± .14	58.5

*Bromphenol blue dye.
†That is, Column 3 plus Column 4.
‡To conserve space, only the average ± ranges are presented.

F. Elution. On each paper strip, components of the albumin pattern, consisting of (1) the major albumin mass, or ('band'), and (2) the albumin trail, were identified by use of the densitometer pattern and a pencil mark made lightly at the junction of the two. The length of the trail from point of origin to junction with the major albumin mass was measured and the area of the trail (in square centimeters) was calculated for each strip.

The 8 strips resulting from one set were then separated into 4 pairs of patterns with identical lengths of the trail and each pair treated in the following manner: The entire pattern of one strip was cut at right angles to the long axis of the paper into short segments prior to elution. The pattern of the other strip of the pair was divided at the point of junction of the albumin band and the albumin trail. Each of the band and trail portions was also cut at right angles to the long axis of the strip into short segments for elution. For blanks, sections of the remaining filter paper strips of exact length as the entire albumin patterns were cut into segments and treated in an identical manner as the filter paper strips containing the albumin patterns. Each group of segments (band, trail, or combined) and the corresponding blanks were eluted 3 times with 5 ml. of 0.5 per cent sodium carbonate (anhydrous) in distilled water (w/v). For the higher concentrations of albumin, modification of the above method was necessary and elution was made using 5 aliquots of 3 ml. each of 0.5 per cent sodium carbonate. Further elutions did not increase the yield of dye.

The eluates were read on an Evelyn colorimeter using 580 mμ filter against the filter paper blank. The resultant values were then compared to a dye standard curve which was obtained by reading solutions of known concentrations of bromphenol blue in 0.5 per cent sodium carbonate in distilled water (against a 0.5 per cent sodium carbonate blank).

G. Adsorption of albumin on filter paper previously saturated with albumin. The strips were prepared by applying 0.010 ml. of 2.66 per cent albumin solution to 4 alternate strips of Whatman No. 3 paper in the prepared electrophoresis cell. Current was then applied to all 8 strips (4 with applied albumin, and 4 control strips) for 6 hours at 7.5 mA. With the strips remaining in the cell, 0.010 ml. of 2.66 per cent albumin solution was then applied to all 8 strips. Electrophoresis was continued for an additional 16 hours. Staining and elution of the strips were performed as previously described.

Results

In Table I are presented the results of the elution studies of varying concentrations of purified human serum albumin subjected to electrophoresis and bromphenol blue staining. It is evident in both thicknesses of strips that the absolute quantity (column 4) of dye eluted from the trail decreases with progressive decrease in the total quantity of albumin applied. Upon calculation of the dye obtained per square centimeter of trail (column 7) which corrects for slight variations in total length of the patterns, the magnitude of the dye bound per unit area is again shown to correlate with the concentration of the applied albumin. There is, however, in the Whatman No. 3 strips, an apparent plateau for dye bound per square centimeter between 162 and 330 μg of albumin, but with both higher and lower concentrations of albumin, the dye bound per unit area shows correlation with the total quantity of albumin applied. No such plateau was observed for the Schleicher & Schuell strips.

In Table II are presented the results of electrophoresis and elution studies of purified human serum albumin from filter paper strips which previously had been treated with human serum albumin. A significant increase of dye bound per unit of path was found in those strips to which albumin had been previously adsorbed. Thus, although diminished, the loss of albumin in the trail was not prevented by the prior adsorption of albumin to the paper.

Table II. Comparison of results of elution studies upon a standard solution (2.66 Gm. %) of human serum albumin migrating by electrophoresis through alternate control strips versus strips previously adsorbed by electrophoresis with human serum albumin solution (utilizing Whatman No. 3 paper)*

Number of Strips	*Column 1.* *Amount of dye† in "band" (μg)*	*Column 2.* *Amount of dye† in "trail" (μg)*	*Column 3.* *Amount of dye† in "band" and "trail" (μg)*	*Column 4.* *Area of "trail" (cm.²)*	*Column 5.* *Dye† per cm.² "trail" (μg)*
A. Control					
4	82.0 ± 5.0	14.5 ± 1.5	97.0 ± 5.0	17.5 ± 1.9	0.83 ± 0.66
B. Albumin-saturated strips					
4	113.0 ± 3.0	18.0 ± 5.0	131.0 ± 8.0	16.3 ± 2.8	1.11 ± 0.14

*To conserve space, the average ± ranges are presented.
†Bromphenol blue dye.

Densitometric patterns indicated a rough correlation with quantity of albumin applied. However, in this study, quantitation was possible only with the elution studies on the same strips.

Discussion

Rigid standardization of the technique of paper electrophoresis has eliminated many of the potential variables in the method and reproducible results may be attained. However, certain factors cannot be avoided by present techniques and are responsible for significant errors in the quantitation of the resultant paper patterns when compared with free, moving boundary electrophoresis. These factors relate to the characteristics of serum proteins in the techniques employed and include: (1) the variable dye-binding capacities of the different globulin fractions when compared with serum albumin[13-19]; (2) the deviation from linearity of dye-binding at high concentrations of albumin[17-20]; and (3) the irreversible adsorption of albumin by the filter paper in its path. The first two of these factors have been discussed previously[13-20] and corrections for these have not been resolved satisfactorily by methods in current usage. The third significant characteristic of serum proteins when separated by electrophoresis on filter paper relates to protein adsorption by the filter paper. The loss of albumin by adsorption not only reduces the ultimate quantity ascribed to this species but also erroneously elevates the globulin fractions (with the exception of the gamma globulin) by the presence of the albumin deposited in the trail.

For a constant trail, correction factors can potentially be introduced to suitably correct the values for the different protein fractions. Previously, certain of the investigations[2-6] indicated that the adsorbed albumin is constant irrespective of the absolute quantity of albumin applied. The constancy of this phenomenon has been reported with bromphenol blue.[2, 4-6] However, recent

studies[7, 8] reported an inconstant loss of albumin in the trail with progressive increases of loss as the concentration of the albumin applied was increased. Since these observations were by densitometry only, the data include the possible variable attributable to the inherent limitations of photoelectric scanners in recording the small quantity of dye in the trail. The present report presents data obtained by direct elution of the stained patterns. For both of 2 standardized laboratory methods of paper electrophoresis, as the albumin concentration was increased, the quantity of protein-bound dye adsorbed per unit area of the filter paper increased in approximately linear fashion. The previously noted[4, 8] increased adsorption of albumin with increased thickness of the filter paper was again observed. Further confirmation of the variability of the trail was shown by the data on the albumin trail obtained after prior adsorption of albumin to the filter paper. A significant increase of the adsorbed albumin over the control strips was observed. Thus, under the experimental methods used in this study, adsorption of a limited, maximal quantity of albumin was not demonstrated.

The explanation for the differing data on albumin adsorption found in the present study from those reported earlier[2, 4-6] is not readily apparent. Samples of human serum albumin were utilized in the present and previous studies.[2, 4-6] Elution of the protein-bound dye, bromphenol blue, was also common to the present and to certain of the previous studies.[2, 4, 6] One difference of possible significance relates to the use of only one aliquot of eluting solvent in the previous investigations,[2, 4, 6] while in the present report, either 3 or 5 aliquots of eluting solvent were used. In our preliminary studies, complete elution could not be achieved by one aliquot of eluting fluid, thus, several aliquots of eluting fluid were employed routinely. Further elution did not increase the yield of dye. The technique of elution appears to be the only discernable difference between the previous studies and the present procedure.

On the basis of the data presented, it would appear that the variable loss of albumin in the trail precludes the adoption of constant correction factors by which the value for the albumin fraction can be elevated and the globulin fractions can be lowered to result in more realistic quantitation of the fractional values of each serum protein component. Instead, a more accurate procedure is the establishment of adequate normal ranges for specific paper electrophoretic techniques and by such data deviations from accepted normal ranges can be readily detected. This approach appears to be more practical for the present, until further modifications in the technique permit individual quantitation of the separated protein species.

We wish to acknowledge the advice and assistance given by Dr. Makepeace U. Tsao throughout this project and during the preparation of this manuscript, and also the suggestions given by Dr. Vernon N. Dodson, who kindly reviewed this manuscript.

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Proceedings of the
CENTRAL SOCIETY for CLINICAL RESEARCH

Thirty-fourth annual meeting

Chicago, Ill., Nov. 3 and 4, 1961

Abstracts

1. Vascular responses of hypertensive patients before and during thiadiazine therapy

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The vascular responses in the forearm and hand were studied in 15 hypertensive patients, using venous occlusion plethysmography, first during a control period and then after several months of thiadiazine, benzydroflumethiazide. In each experimental period, data were collected at rest and during and/or following 5 minutes of arterial occlusion, 30 seconds of local anaerobic work in the forearm, and 30 minutes of exposure to a bath temperature of 45° C. In most instances the results were compared with those obtained in 17 normotensive subjects.

The average resting forearm blood flow in the hypertensive group was 3.0 ml. per minute per 100 ml. limb volume, as compared with 1.5 ml. for the normotensive subjects, the difference being significant ($p < 0.001$). The average resting hand blood flow was 7.3 ml., as compared with 7.5 ml. for the normotensive group, the difference not being significant.

After an average of 3 months of therapy the resting blood flow in the forearm fell to 2.4 ml., while that in the hand was 6.8 ml., the change in the forearm being found significant ($p < 0.05$).

The reactive hyperemia response in the forearm to a period of local anoxia was much more marked in the hypertensive than in the normotensive subjects (average peak rise of 14.9 ml., as compared with 7.7 ml.; and a total excess blood flow of 11.4 ml., as compared with 7.2 ml.). A similar type of relationship was noted with wet heat (average peak rise of 7.8 ml., as compared with 5.4 ml.; and a total excess blood flow of 164.0 ml., as compared with 108.0 ml.).

After therapy, no significant alterations occurred in the vascular responses to local anoxia and wet heat in the forearm and hand and to anaerobic exercise in the forearm.

In conclusion, the resting blood flow in the forearm of hypertensive patients was found to be increased, as were the vascular changes elicited by local anoxia and wet heat in this site. Except for a drop in resting forearm blood circulation, the prolonged administration of thiadiazine therapy did not alter the responses in the hand and forearm, despite a resulting fall of 33 mm. Hg in systolic and 11 mm. Hg in diastolic blood pressure.

2. On the mechanism of aminonucleoside nephrosis in rats

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The aminonucleoside (P-A) of puromycin [9-(3-amino-3-deoxy-β-D-ribofuranosyl)-6-dimethylaminopurine] regularly produces nephrosis in rats by a mechanism not yet understood. The delayed appearance of proteinuria in contrast to its immediate appearance in nephrotoxic serum-treated rats suggested the involvement of an immune mechanism. However, cortisone or nitrogen mustard administration, complement fixation, precipitin tests, and passive transfer experiments have all failed to provide evidence for an immune mechanism.

Simultaneous administration of many known ribonucleic acid precursors as well as antimetabolites (5-fluorouracil, 6-mercaptopurine) did not block or reverse P-A induced nephrosis, except adenine, which offered only partial and temporary protection. Weekly or biweekly administration of P-A at levels ranging from 1.5 mg. per 100 Gm. to 0.5 mg. per 100 Gm. induced proteinuria at a much less total cumulative dose than when administered in the usual daily dose of 1.5 mg. per 100 Gm., suggesting a mechanism of action similar to that of certain carcinogens.

Radioactive P-A (8-C^{14}) was administered to 3 rats, 2 of which received 3 and 13 doses, respectively, of unlabeled P-A prior to the 8-C^{14} compound. Expired air, urine, and feces were collected for the next 8 hours, after which the animals were sacrificed. Various organs were homogenized and counted in Hyamine hydroxide with the use of a liquid scintillation counter. Except for the 13 day rat, which was frankly nephrotic, 65 to 70 per cent of the radioactivity of 8-C^{14}-labeled P-A was excreted in the urine 8 hours after subcutaneous administration, while 15 to 26 per cent appeared in the feces. Unchanged P-A accounted for 55 per cent of the urinary radioactivity as determined by inverse isotope dilution. The nephrotic rat excreted only 30 per cent of the radioactivity in the urine and 13 per cent in the feces, presumably as a result of the generalized state of water retention. These data argue against any mechanisms involving *direct* enzyme block.

The evidence to date favors either (1) incorporation of P-A itself into nucleic acid, which could result in replication of basement membrane of altered physical-chemical structure, or (2) interference with protein synthesis, thus preventing the synthesis of normal basement membrane at a normal rate.

3. Pneumococcal lobar pneumonia, the pulmonary circulation, and anoxia

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In 35 patients with unilateral uncomplicated pneumococcal lobar pneumonia and in 8 with complicating pleural effusion, only modest arterial O_2 unsaturation (93 per cent ± 3 and 92 per cent ± 3) was observed, despite extensive pulmonary consolidation. These observations imply absent or reduced perfusion of unaerated lung segments. That arterial O_2 unsaturation is minimized in these patients by such a mechanism was indicated by bronchospirometric studies showing that the reduction in O_2 uptake, and presumably, therefore in blood flow, of the affected lung corresponded well with the amount of anatomic involvement.

Further investigations of mechanisms accounting for the observed mild arterial O_2 unsaturation supported the hypothesis of corresponding reduction in perfusion and ventilation of consolidated lung segments. These included (1) nitrogen washout studies of intrapulmonary

gas mixing, (2) arterial blood gas studies during low O_2 breathing, and (3) balloon catheter occlusion of the pulmonary artery supplying the affected lung. Intrapulmonary gas mixing was essentially the same in the affected and unaffected lungs, and both were normal. The increased alveolar arterial O_2 tension difference on room air was reduced regularly during low O_2 inhalation to a normal value, indicating a venous admixture effect rather than a diffusion defect as the mechanism for reduced arterial O_2 tension and saturation. Obstruction of pulmonary arterial blood flow to the affected lung caused no significant change in arterial O_2 tension. Thus the venous admixture effect was not brought about by perfusion of unaerated portions of lung. It has been concluded that pneumonic consolidation is accompanied by corresponding reduction or cessation of perfusion in unaerated segments, and that fringe areas of unequal ventilation and perfusion seem not to occur. It is hypothesized that the venous admixture effect resulting in mild arterial O_2 unsaturation may be related to changes in bronchial circulation with increased bronchial venous drainage to pulmonary veins.

4. Studies of the metabolism of human transferrin

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Crystalline human transferrin was labeled with radioactive iodine and injected into normal subjects and patients with a variety of diseases in order to study the metabolism of this iron-binding protein, particularly in relation to concomitant measurements of radioiron turnover. The integrity of the transferrin after iodination was evaluated by the following procedures: (1) electrophoretic mobility; (2) spectrophotometric iron-binding properties; (3) Ouchterlony gel diffusion; (4) ultracentrifugation; (5) immunoelectrophoresis; (6) absorption spectrum; and (7) biologic testing in patients. Following preliminary ferrokinetic measurements, subjects were given 20 to 100 mg. of transferrin labeled with 40 to 150 μc of radioiodine, and measurements of I^{131} activity in plasma, urine, feces, and gastric juice were made at intervals for periods of 20 to 30 days. Plotted semilogarithmically, plasma radioactivity decreased in a curvilinear manner for 4 to 6 days and thereafter assumed a linear disappearance.

Eight normal men showed the following: Half-time plasma transferrin clearance of 8.0 to 10.4 days; total body transferrin of 7.8 to 14.6 Gm., with almost equal distribution between vascular and extravascular compartments; and mean transferrin degradation rate of 11.5 mg. per kilogram per day.

Ten anemic patients were studied to observe the effect of altered erythropoiesis and iron turnover on transferrin metabolism. Prolonged plasma half-time transferrin clearance was found in 4 patients with iron deficiency anemia, especially in one patient with iron deficiency and myxedema; shortened clearance values were obtained in patients with other types of anemia (hemolytic, hypoplastic, and pernicious anemia). Normal transferrin metabolism was found in a patient with hemochromatosis. Erythropoiesis was altered in patients with sickle cell anemia by repeated packed erythrocyte transfusions, in pernicious anemia by vitamin B_{12} therapy, and in iron-deficient patients given therapeutic doses of iron; no appreciable effect on transferrin metabolism was observed. The distribution of radioiodine in the various organs and secretions of the body was explored and highest concentrations were found in the thyroid and salivary glands and in the proximal portion of the gastrointestinal tract. No correlation of transferrin metabolism with measurements of erythropoiesis or iron turnover was found.

5. Pulmonary abnormalities in congestive heart failure

GEORGE N. BEDELL, M.D., YUTARO SUZUKI, M.D. (By Invitation), and WILLIAM R. WILSON, M.D. (By Invitation) *Iowa City, Iowa*

Lung function studies are reported in 9 patients with left ventricular failure, 6 with rheumatic heart disease, and 3 with coronary artery heart disease. All had cardiomegaly, pulmonary congestion, and hepatomegaly; most had peripheral edema and pleural effusion. Studies were done shortly after hospitalization and repeated after significant clinical improvement, usually 10 or 15 days later.

Table I. Lung function studies: Mean values before and after therapy

Function	*Before*	*After*
Vital capacity (ml.)	2,200	2,830
Residual volume (ml.)	2,210	2,345
Minute volume of alveolar ventilation (L.)	10.2	9.1
Alveolar gas distribution	Normal	Normal
Physiologic dead space (ml.)	254	219
Per cent of tidal volume	49	47
Maximal breathing capacity (L./min.)	52	70
Maximal expiratory flow rate (L./min.)	111	183
Diffusion (ml. CO/mm. Hg/min.)	18	18
Arterial blood studies		
O_2 saturation (%)	93.8	95.0
PCO_2 (mm. Hg)	35	37
pH	7.45	7.46

The patients probably have edema of the tracheobronchial tree and of the alveolar capillary membrane to produce the reduced mechanical function and diffusing capacity. The finding of normal distribution of inspired air and increased physiologic dead space suggests uneven distribution of blood flow in the lungs.

6. Serum lipid and fat tolerance determinations in patients receiving Sippy diets

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Introduced by John H. Moyer, M.D.

The rationale for the use of the high fat Sippy diet in the treatment of peptic ulcer has been well substantiated since its introduction in 1915. Objective measurements of gastric motility plus subjective clinical response attest to its value in the patient with active symptoms.

The recent evidence aligning coronary atherosclerosis with dietary intake of fats, particularly of the saturated variety, plus data suggesting an increased incidence of myocardial infarction in ulcer patients, now makes it necessary to reappraise the Sippy diet in terms of its possible deleterious implications.

To study this problem further, the serum triglycerides and cholesterol plus the I^{131} triolein tolerance were measured over an 8 hour period during the administration of a Sippy diet in 2 groups of patients with active peptic ulcers. In one group the ulcer disease was the only disorder. In the other, an additional condition thought to be associated with a dysfunction in lipid metabolism, i.e., coronary artery disease, xanthelasma, xanthomatosis, or hypercholesterolemia, was present.

The results showed that in every case a persistent elevation of the triglycerides prevailed during the period of administration of the milk-cream feedings. In the group with a lipid disturbance already present, the hyperlipemia was further exaggerated. Radioactive fat tolerance likewise was abnormal in each patient, the degree of impairment being proportional to the height of the triglyceride concentration. Cholesterol levels did not show any marked alterations.

It is postulated that prolonged intake of the Sippy diet in patients with peptic ulcers may lead to lipid biochemical alterations that are conducive to the development of atherosclerosis and its complications.

7. Use of a digital electronic computer for evaluation of serial clinical observations in acute leukemia

WILLIAM R. BEST, M.D. *Chicago, Ill.*

The critical evaluation of therapeutic agents in certain diseases is best accomplished through the cooperation of several institutions utilizing detailed protocols relative to patient management and appraisal. Such investigations produce a mass of clinical and laboratory data which represent repeated examinations of many patients and which must be laboriously reviewed to detect deviations from protocol and to derive specific summary data regarding individual patient visits and patient courses under therapy. The Midwest Cooperative Chemotherapy Group is comparing 3 methods of therapy in adults with acute leukemia. A computer program to accomplish the necessary data review and evaluation for this study was written for the Bendix G-15 digital computer.

The computer accepts detailed data from one patient visit at a time, rates the status of bone marrow, blood, physical findings, and symptoms, as well as the total disease status at that visit; considers each visit in terms of prior visits; indicates any of 12 possible deviations from protocol; indicates at which visit criteria for removal from study have been fulfilled; indicates which of 6 categories of response to therapy characterizes the total course of each patient; and prepares a summary table indicating all changes in disease status and drug dosage which occurred during a patient course. Additions to the program could be constructed to analyze and summarize data from all patients in a study. In the future it might be expedient for each investigator to mark with a conducting pencil in the appropriate boxes of a patient-visit check-sheet. A machine would then convert the data from that visit directly onto a punch card for later entry into the computer.

A number of solutions to pertinent problems in programing were developed. For example, all dates are converted by the computer to an absolute scale which takes into account the number of days in specific months and years. An anemia index permits hemoglobin and hematocrit values of either sex to be analyzed interchangeably.

The feasibility of using computers to check adherence to protocol and to summarize patient data has been demonstrated. Increasing use of computers for similar purposes is predicted.

8. Hypertransfusion-induced gastrointestinal ulceration: Species and dose factors

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Introduced by R. J. Hasterlik, M.D.

An earlier report from our laboratory described gastric ulceration in rats hypertransfused with red cells. The present studies were undertaken to observe this phenomenon in other

species and to determine the frequency of this lesion as related to the dose of injected erythrocytes.

Two strains of mice, CF-1 and C-57, were hypertransfused with isologous red cells so that hematocrit values of 65 to 75 per cent were attained. In none of the 50 mice treated was gastrointestinal ulceration observed.

Swift rabbits were given homologous red cells until hematocrit readings of 70 to 75 per cent were achieved. Autopsy revealed ulceration with hemorrhage in the stomach and/or proximal intestine in 23 of 46 hypertransfused animals.

Inbred Lewis and WR strain rats were studied to test the possibility that ulceration might be a manifestation of interstrain immunologic incompatibility. Eleven of 18 Lewis rats and all 5 WR rats exhibited typical gastric ulceration after administraton of isologous red cells.

Sprague-Dawley rats were used for evaluation of the dose of injected erythrocytes as a factor. When one injection of red cells equivalent to 2.6 per cent of body weight was given, a mean maximal hematocrit of 62.1 per cent was reached, and 2 (14 per cent) of 14 hypertransfused rats showed gastric ulceration. Two injections of red cells, totaling 5.3 per cent of body weight, were followed by a mean maximal hematocrit of 66.9 per cent, and ulceration of the stomach in 4 (25 per cent) of 16 animals. Three injections of erythrocytes, equivalent to 8 per cent of body weight, raised the mean maximal hematocrit to 73.1 per cent, and gastric ulceration was observed in 25 (81 per cent) of the 31 hypertransfused rats.

The occurrence of intestinal as well as gastric ulceration in the hypertransfused rabbit, and the absence of this phenomenon in the similarly treated mouse indicate the importance of species factors in the production of these alimentary tract lesions. The increasing frequency of gastric ulceration with larger amounts of transfused red cells in the rat suggests a dose-dependent relationship.

Although the dose-response results in the rat may have some bearing on the pathogenesis of peptic ulcer in polycythemic patients, the species variations described call for caution in interpretation of these data.

9. Effect of acetyl strophanthin on myocardial electrolytes in dogs with congestive heart failure

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Introduced by Robert C. Muehrcke, M.D.

Studies were made on the effect of a rapidly acting esterified cardiac aglycone on the electrolyte, nitrogen, and water content of the normal and failing dog myocardium. The syndrome of chronic congestive heart failure was produced in female dogs by progressive pulmonary artery constriction. A factorial experimental design, containing 7 replicates of 4 dogs each, was employed. Each replicate was completed in 1 day and consisted of 2 groups of 2 dogs each, one group containing dogs with heart failure and the other being made up of normal female dogs. One animal from each group received intravenous acetyl strophanthin, 0.015 mg. per kilogram ascites-free weight, while the other was given an injection of inert material. The heart was removed from the chest quickly 9 minutes after the injection and the right ventricle was analyzed for its constituents.

Previous data indicated that this dose of the drug produced an inotropic effect in both normal animals and dogs with heart failure without producing digitalis toxicity. The myocardium from animals with congestive heart failure contained less potassium* and nitrogen* but more sodium,* chloride,* and water* (per kilogram of dry fat- and blood-free myocardium) than the myocardium from normal dogs. Acetyl strophanthin increased sodium,† chloride,* and water* in both groups of dogs, but failed to change nitrogen in either group.

*Significant at the 0.005 level.
†Significant at the 0.05 level.

Although potassium was unchanged in treated control dogs, it was increased* by acetyl strophanthin in animals with heart failure.

The sodium, chloride, and water accumulation produced by this digitalis-like compound is consistent with inhibition of active sodium transport out of myocardial cells in both groups of animals. The effect of acetyl strophanthin on potassium suggests a unique and possibly reparative action of the drug on the failing heart.

10. Factors influencing the late secondary rise in the plasma nonesterified fatty acids following glucose loading

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The plasma nonesterified fatty acids (NEFA) respond to glucose administration by decreasing. Four to 6 hours after the glucose administration there is a rise in the NEFA to values significantly higher than the original fasting values. The factors influencing this secondary rise in the NEFA were studied in 28 subjects to whom a glucose load of 50 Gm. had been administered intravenously. A correlation of 0.86 was noted between the degree of hypoglycemia occurring after the initial glucose peak and the magnitude of the secondary rise in the NEFA. Catacholamine production was studied during the glucose tolerance test by measuring the output of vanil-mandelic acid (VMA) at hourly intervals. A significant increase over fasting values occurred in the VMA excretion in all cases. The magnitude of the VMA peak showed a correlation of 0.55 with the secondary rise of the NEFA.

There was a significant increase in the excretion of 17-hydroxycorticoids during the glucose tolerance test but the magnitude of this did not correlate with the secondary rise in the NEFA.

Plasma insulin-like activity at the fourth hour was measured by the epididymal fat pad method. There was a correlation of 0.60 between this value and the secondary rise of the NEFA. A correlation of 0.86 existed between the insulin-like level and the magnitude of the VMA peak.

The secondary rise in the NEFA was found to be exaggerated in a case of Cushing's disease and a patient with pheochromocytoma. It was absent in a patient who had undergone bilateral adrenalectomy.

Thus the degree of hypoglycemia most closely determines the extent of the secondary NEFA rise after glucose. This effect is mediated in part through a catacholamine response to insulin production.

11. The lack of species specificity of antibody to an enzyme

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Serum factors which react with nuclei, mitochondria, microsomes, and other cell components of many species occur in systemic lupus erythematosus and other diseases. The observation that antimitochondrial sera can inhibit enzymes in these particles suggested a possible basis for lack of species specificity. Enzymes from different sources with similar properties, including substrate specificity, pH optima, and kinetic characteristics, presumably have similar active sites. Antienzymes may cause inhibition by reacting with active sites; in such instances, the antibody might be expected to react with the same sites on similar enzymes from other species.

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*Significant at the 0.005 level.

To test this hypothesis, antiserum was obtained from rabbits following injection of crystalline bovine hepatic L-glutamic dehydrogenase (GDH). These sera inhibited the bovine GDH. They also inhibited hepatic GDH prepared from rat, rabbit, human, frog, and pigeon. Frog kidney GDH, which is similar to the hepatic enzymes in requiring either di- or triphosphopyridine nucleotide as a cofactor, was also inhibited. Frog muscle GDH, which requires diphosphopyridine nucleotide, was similarly inhibited; yeast GDH, which requires triphosphopyridine nucleotide, was not inhibited by this antibody. Inhibition occurred with or without the inclusion of complement and was most marked when the least enzyme was used in the assay. These sera did not inhibit lactic dehydrogenase.

Kinetic studies suggested that the anti–bovine GDH caused mixed competitive and non-competitive inhibition of the bovine enzyme, suggesting reaction with multiple sites on the enzyme protein. With GDH of other species, kinetic data suggested competitive inhibition, pointing to a more specific reaction with active sites on the enzyme.

These findings indicate that antibodies to an enzyme can inhibit similar enzymes from other organs and other species. The lack of species specificity of lupus serum factors may be related to this phenomenon.

12. The correlation of blood and fecal radioactivity after oral administration of I^{131} triolein

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The determination of radioactiviy in venous blood after ingestion of I^{131} triolein has proved to be a valuable test for assessing fat absorption. However, in instances of mild impairment of fat absorption the measurement of radioactivity in venous blood may not detect the abnormality. In an attempt to improve discriminating ability of the "I^{131} triolein absorption test," we have made a critical analysis of blood and fecal radioactivity values in 156 consecutive tests. Parameters considered in the analysis of data included peak concentration of radioactivity in venous blood, the area described by curve when concentration of radioactivity of venous blood was plotted against time, and the total radioactivity recovered in feces.

The most discriminating values for the 3 parameters were 10 per cent or greater of administered dose for blood peak concentration, 5 per cent or less of administered dose for total stool concentration, and 20 cm.^{2} for the 6 hour blood area.

Applying these values for "normal" to the results obtained when I^{131} triolein was administered to 27 patients known to have fibrocystic disease and 14 normal subjects, the total blood area (6 hour) and total stool concentration discriminated normal from abnormal pancreatic exocrine function better than peak blood concentration. No values for the 3 parameters could be determined to discriminate completely known normal from abnormal pancreatic function.

13. Regeneration of colon mucosa: A morphologic and histochemical study

RICHARD E. BRAUCHER, M.D. (By Invitation), and JOSEPH B. KIRSNER, M.D. *Chicago, Ill.*

This investigation in 50 full-grown dogs was undertaken to develop a simple experimental model for study of the process of regeneration of colon mucosa and to delineate some of its aspects. Because the ulcers in chronic ulcerative colitis most frequently are localized at or above the muscularis mucosae, approximately 1 cm.^{2} of mucosa was excised immediately superficial to muscularis mucosae via laparotomy and antimesenteric colon incisions. The muscularis mucosae was excised in some lesions. Postoperative autopsies were done at intervals ranging from 6 hours to 12 weeks.

The lesions were studied by hematoxylin and eosin, periodic acid–Schiff, Gomori trichrome, and silver reticulum techniques. Rates of regeneration varied. A flat to cuboidal single-cell layer appeared at 24 to 48 hours, covering as much as one half the defect by the fifth day. Gradually increasing degrees of glandular formation, with branching, corkscrew, and cystic configurations, were observed. By the thirteenth to twenty-first days, the lesion was completely covered by at least a single layer of cells, much of it with advanced gland formation. Eventually, the glands regained a normal appearance. Intracellular periodic acid–Schiff–positive material was absent before glandular re-formation. Dense connective tissue and increased vascularity were noted in the submucosa. Regeneration of mucosal reticulum was demonstrated. Excised muscularis mucosae did not regenerate and glandular formation appeared retarded in such lesions.

Histoenzyme studies in frozen sections showed apparent decrease in succinic dehydrogenase activity, subsequently returning to normal. The proliferating single layer demonstrated increased β-glucuronidase activity. This returned to normal or decreased in the deeper portions of the regenerating glands adjacent to the single layer of cells.

These observations demonstrate complete mucosal regeneration in lesions superficial to muscularis mucosae. Periodic acid–Schiff–positive mucopolysaccharide and succinic dehydrogenase activity are temporarily absent or diminished, while β-glucuronidase activity increases initially. The absence of permanent changes in this type of lesion suggests other factors besides simple destruction of mucosa in the pathogenesis of chronic ulcerative colitis.

14. The influence of alkylating agents on the ratio of target to nontarget uptake of P^{32} in rat neoplasm

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Several human malignancies concentrate P^{32} appreciably. The chief drawback to therapeutic use is simultaneous uptake by nontarget organs—most critically, bone marrow. An agent increasing tumor to marrow uptake *ratio,* whether by increasing tumor uptake or decreasing marrow uptake, should improve the therapeutic usefulness of P^{32}. Among possible agents for influencing this ratio are chemotherapeutic drugs. Moreover, development of tumor resistance to the drug's chemotherapeutic effect might not destroy its ability to improve uptake ratio; it might even increase this ability if drug depression of tumor P^{32} uptake waned more than the ability of the drug to protect marrow from uptake. Drugs used in this adjunctive way might thus retain usefulness even against tumors resistant to conventional use of the drug.

These possibilities were explored in rat Flexner-Jobling carcinosarcoma. Thio-TEPA (10 mg. per kilogram) or Triethylenemelamine (1 mg. per kilogram) was given subcutaneously 29 hours before sacrifice; control subjects received saline. P^{32} (20 μc as phosphate) was injected intramuscularly 5 hours before sacrifice. Femoral marrow and blood specimens were dried in planchets and counted in an end-window counter. Homogenized tumor was counted in a well counter before drying or dried, dissolved in HNO_3 and counted in planchets after redrying. All counts were compared with similarly counted standards. Appropriate smears and histologic sections were obtained.

In 12 control and 13 treated rats, mean uptakes were as follows, respectively: tumor, 3.58 (per cent administered dose per gram dry weight) and 3.05; marrow, 1.92 and 0.48. Respective tumor to marrow mean uptake ratios were 1.98 $\pm$ 0.28 (standard error) and 8.55 $\pm$ 2.16, a highly significant difference ($p < 0.01$).

Other rats bearing this tumor received 12 daily thio-TEPA (½ mg. per kilogram) doses to promote resistance. A strain derived from tumor surviving this pretreatment was transplanted and studied in 11 rats as above. Respective mean uptakes after saline and thio-TEPA were as follows: tumor, 3.74 and 3.63; marrow, 2.61 and 1.06. Respective uptake ratios were 1.43 ± 0.38 and 5.32 ± 1.78 ($p = 0.09$).

The drugs favorably affected tumor to marrow P^{32} uptake ratio in nonresistant tumors. The data suggest this favorable effect may not be abolished in tumor surviving conventional treatment with such drugs.

15. Decreased glutathione reductase with susceptibility to hemolysis

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Introduced by A. S. Alving, M.D.

Glutathione reductase (GSSG-R) is increased in human erythrocytes deficient in glucose-6-phosphate dehydrogenase (G-6-PD) and susceptible to primaquine-induced hemolysis (J. LAB. & CLIN. MED. 52: 109, 1958). Long and Carson (Biochem. & Biophys. Res. Comm. 5: 394, 1961) report that in erythrocytes of diabetics GSSG-R is also high but G-6-PD is normal.

We have recently investigated blood from a Caucasian volunteer with low GSSG-R and normal G-6-PD. He has proved to be susceptible to hemolysis induced by 8-aminoquinolines several times during the past 15 years. The following results have been obtained on his blood: the GSSG-R is 57 per cent of normal—almost 3 standard deviations from the mean; the erythrocyte reduced glutathione (GSH) is low but the GSH stability test is negative; glucose utilization by the erythrocytes incubated with methylene blue is normal, which suggests normal function of the pentose-phosphate pathway.

The Cr^{51}-labeled erythrocytes of this volunteer were transfused into normal recipients. In the recipients given 14 days of quinine, sulfoxone, isopentaquine, primaquine, or isopentaquine with quinine, the Cr^{51} half-lives of the transfused cells were 23.5, 21.0, 18.5, 17.0, and 16.5 days, respectively. Control half-life was 27 days.

In contrast to erythrocytes with high GSSG-R and deficient G-6-PD, erythrocytes with low GSSG-R and normal G-6-PD are also susceptible to drug-induced hemolysis. This has been predicted; Carson (Fed. Proc. 19: 995, 1960) and DesForges and co-workers (Am. J. Med. 27: 132, 1959) have reported a case of hemolytic anemia induced by sulfoxone in which there was a "slight reduction of GSSG-R activity." The degree of deficiency in our case is not sufficient to demonstrate failure of the pentose-phosphate pathway by the in vitro tests so far applied (a severe or total deficiency of GSSG-R may be lethal). Moreover, evidence is accumulating from many laboratories that the pentose-phosphate and glycolytic pathways interact and this may be mediated by the reactions of glutathione as well as the pyridine nucleotides. Thus, participation of the glycolytic pathway in this hemolytic process must also be postulated. We regard the susceptibility to hemolysis of this individual with low GSSG-R as further evidence for a relationship of changes in GSSG-R to lesions of carbohydrate metabolism.

16. Estimation of the tissue work of the lung

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Introduced by Harry F. Dowling, M.D.

The static compliance of the human lung is due to the action of surface forces at the air-fluid interface and of tissue forces in the walls of the terminal air spaces. The behavior of the former is becoming increasingly better understood; the role of tissue elements

(especially elastic tissue and collagen) is still uncertain. Much of this uncertainty could be resolved if the pressure-volume curves of the lung could be interpreted in terms of the action of connective tissue fibers.

We have previously demonstrated experimentally that single isolated elastic fibers from one mammal (the ox) show a constant exponential relationship between an applied force and the resulting stretch obtained. It is possible, therefore, to calculate the work done in stretching this type of tissue; all parts of the relationship

$$\text{Work} = \int F \, ds$$

(where F = force and s = length) are known. The geometric form of the lung is such that volumes change as the cube of the change in length of linear elements, such as the circumferences of the alveoli. If one assumes that elastic tissue is a simple tissue and that its stretch properties are similar from one mammalian species to another, then the tissue work performed in stretching an alveolus or a lung can be calculated and compared with that measured by independent means using pressure-volume curves. This has been done and shows the following: (1) the calculated tissue work-volume curves of the lung are similar in form to those measured experimentally; (2) the tissue work of the lung is dependent upon the mass of elastic tissue in the walls of the alveoli and is independent (in this ideal situation) of the size and number of alveoli; (3) the human lung is estimated from these assumptions as having a total capacity of 6 L., in agreement with observation; (4) the tissue work of the lung can be interpreted in terms of the basic stretch properties of elastic tissue.

17. Changes in metabolism of sodium, potassium, and water in acute hepatitis

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Although disorders of water and electrolyte metabolism are commonly associated with severe hepatic disease, especially cirrhosis with ascites, their relationship to liver cell failure is uncertain as opposed to secondary disorders of endocrine and renal function, or portal hypertension. To define the changes accompanying mild hepatic dysfunction, determinations of total body water, exchangeable sodium, exchangeable potassium, and serum electrolyte concentrations were made in 9 patients with uncomplicated acute "viral" hepatitis. The results early in the icteric phase were compared with the findings after recovery (mean interval between studies, 5 weeks).

In the posticteric phase, all subjects had gained water, but there was no change in flesh, as reflected by dry body weight. However, absolute increases in total body water, exchangeable sodium, and exchangeable potassium occurred. The gain of sodium paralleled that of water, but the most striking change was the degree of repletion of potassium. Minor changes in serum concentrations of sodium and potassium occurred, and these were unrelated to changes in body water, exchangeable sodium, and exchangeable potassium.

These results were interpreted as follows. Depletion of water, potassium, and sodium during the preicteric and early icteric phases of hepatitis reflect anorexia and catabolic processes, the latter being particularly important in relation to the excessive potassium loss. During the posticteric phase, repletion of sodium and water occurs rapidly, together with a striking increase in body potassium stores. The latter reflects restoration of ionized potassium and potassium acquired through anabolism, including glycogen repletion, as no gain in flesh was evident at this stage, perhaps because of continued impairment

of protein synthesis following liver disease. The exchangeable sodium to exchangeable potassium ratio was abnormal during the icteric phase, as a result of a greater loss of potassium than sodium, rather than as a result of sodium retention.

18. An approach to characterizing human connective tissue cells

C. WILLIAM CASTOR, M.D. *Ann Arbor, Mich.*

Clinical difficulties attending the diagnosis and treatment of "self-perpetuating" chronic inflammatory diseases, as rheumatoid arthritis and scleroderma, serve to emphasize the inadequacy of our knowledge concerning the connective tissues. While collagen and several mucopolysaccharides of the ground substance have been studied intensively, the cells themselves have received scant attention. Little is known about the composition, function, or interaction of connective tissue cells. In the present study, connective tissue from several sites was cultured in vitro in order to obtain sufficient cellular material for comparison. Fibroblast-like cells derived from granulation tissue, normal synovium, dermis, periosteum, pleura, and peritoneum were examined by cytologic, chemical, and electrophoretic methods.

Stained coverslip preparations revealed large quantities of periodic acid–Schiff–reactive material in cells of peritoneal and pleural origin. Morphologic parameters, however, generally were inadequate for distinguishing cells from different origins. Mean cell volume measurements disclosed modest differences in cell size.

The rate of cell proliferation, expressed as average generation time, ranged from 60 to 296 hours. Cells from periosteum and granulation tissue grew rapidly, while peritoneal cells multiplied slowly. The rate of carbohydrate utilization was similar for all cell types. On the other hand, the rate of acid mucopolysaccharide synthesis was high for pleural and peritoneal "fibroblasts," intermediate for synovial and dermal cells, and relatively low for cells derived from chronic granulation tissue.

The phospholipid to protein ratio varied from 0.20 to 0.31, except in the case of pleural and peritoneal cells, where it was 0.37. To initiate the qualitative examination of the cellular proteins, the sucrose soluble components of several cell lines were subjected to electrophoresis in a vertical descending starch gel system. Starch blocks stained for protein uniformly showed 12 bands, in addition to 4 "achromatic" bands. Of interest was the regular finding of a protein band with electrophoretic mobility like the slow α_2-globulin described by Smithies in human plasma. Enzyme stains carried out on the starch blocks revealed 2 distinct acid phosphatases, 8 esterases, and essentially no alkaline phosphatase. Enzymatic differences between cell lines appeared to be quantitative rather than qualitative.

19. Fundamental differences of pancreas-stimulating hormones as determined by electrophoresis

JOHN B. CHRISTODOULOPOULOS, M.D. (By Invitation), and
ARTHUR P. KLOTZ, M.D. *Kansas City, Kan.*

Electrophoretic investigation of canine pancreatic juice was performed on basal-, secretin-, pancreozymin-, and cholecystokinin-stimulated specimens obtained from dogs with chronic duodenal fistula. Invariable and distinct differences were found between basal- and secretin-stimulated specimens. Cholecystokinin and pancreozymin specimens differed but were similar to one another. Characteristic electrophoretic patterns were always obtained.

Anticholinergic drugs altered the electrophoretic pattern of pancreozymin and cholecystokinin, whereas carbonic anhydrase inhibitors had no effect on the pattern.

Previous experimental work has shown that pancreozymin and cholecystokinin produce a surprising increase in volume and bicarbonate of pancreatic juice heretofore unappreciated. This new fundamental characteristic is supported by their different electrophoretic patterns and their response to anticholinergic drugs and carbonic anhydrase inhibitors as compared to secretin.

Isolation and characterization of canine pancreatic proteins by ion exchange chromatography are under way.

20. Renal lesions in dogs produced by plasma from patients with systemic lupus erythematosus

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Introduced By Robert B. Leach, M.D.

When plasma from patients with systemic lupus erythematosus is infused intravenously into dogs, a renal lesion develops in the majority of the recipient animals. The dogs in this study were infused once or twice on alternate days with an average of 60 ml. of plasma and sacrificed from 3 to 30 days later.

The renal changes consist of thickening of glomerular capillaries, hypercellularity, hyperemia, and occasional focal lesions resembling "wire loops." Periodic acid–Schiff stains indicate that thickening of basement membrane or marked endothelial cell swelling has occurred. Serial renal biopsies from infused dogs with one kidney explanted into the flank show an evolution of these findings.

Infusion of plasma from normal subjects and patients with other collagen diseases did not produce similar changes. No other consistent renal abnormalities were noted and other tissues were apparently uninvolved.

Fluorescent antibody studies have indicated that a factor in fluorescein-labeled systemic lupus erythematosus plasma adheres to the glomeruli of normal dogs when overlaid directly. The glomeruli of dogs previously infused with systemic lupus erythematosus plasma show some fluorescence when overlaid with fluorescein-labeled antihuman globulin.

These changes suggest that a factor in systemic lupus erythematosus plasma is capable of producing a lesion in the kidneys of the dog resembling that of the disease in the human, and that an immunologic mechanism may be involved.

21. The role of certain nucleosides in carbohydrate metabolism

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There is considerable experimental evidence that the liver exerts an important influence on glucose uptake by peripheral tissues (brain and muscle). It has been postulated that this influence is exerted by way of a "hormonal factor" released by the liver. We have previously reported evidence in man that a derivative of two nucleosides (uridine and cytidine) may be the hypothetical liver factor in question.

The present studies were undertaken to define more precisely the nature of the influence of nucleosides on glucose metabolism. The isolated rat diaphragm was used as a representative peripheral tissue. It was found that the diaphragm from a dehepatized rat did not exhibit a normal response to insulin, i.e., an increased uptake of reducing substance(s) from the incubating medium. Furthermore, the normal response to insulin could be restored by the addition of uridine or cytidine to the medium. These nucleosides

had no significant effect on the uptake of reducing substance(s) by the diaphragm from intact rats. However, it was shown that the uptake of C^{14} glucose by the diaphragm of the dehepatized rats was normal. This suggests that the diaphragm of the liverless rat is peculiarly permeable to some reducing substance(s) within the muscle cell, and that certain nucleosides have the ability to prevent this abnormality. It was also demonstrated that excessive loss of reducing substance(s) from the diaphragm of normal rats was produced by the addition of 2 mg. per cent bilirubin to the incubating medium and that this also could be prevented by the addition of cytidine or guanidine to the medium. These results appear to indicate that the liver plays a major role in maintaining normal muscle cell membrane function.

22. Disparate changes in lipoprotein lipid with the substitution of dietary carbohydrate for animal fat

LOUIS COHEN, M.D. (By Invitation), LJUBICA DOBRILOVIC (By Invitation), and RICHARD J. JONES, M.D. *Chicago, Ill.*

When carbohydrate is substituted for dietary animal fat, Gofman observed that serum cholesterol and lipoproteins of S_f 0 to 20 fall, but lipoproteins of S_f 20 to 400 increase. He inferred that lipoprotein cholesterol changes parallel those of the corresponding lipoproteins, and therefore questioned the value of the serum cholesterol as a guide to the dietary therapy of hypercholesterolemia, and the wisdom of substituting carbohydrate for dietary fat to lower serum cholesterol. The present study was undertaken to assess directly the changes in lipoprotein cholesterol and phospholipid which accompany the substitution of carbohydrate for dietary animal fat.

Five adult men consumed isocaloric diets, constant in protein and cholesterol, for 2 months. For one month the diet was fat-rich and carbohydrate-poor; for the other, the reverse. Blood was collected in the postabsorptive state during each diet period. Whole serum and lipoproteins of density $<$ 1.019 (S_f 12 to 400), 1.019 to 1.063 (S_f 0 to 12), and $>$ 1.063 were analyzed for cholesterol and phospholipids.

When dietary animal fat was replaced with carbohydrate, the whole serum cholesterol fell in 4 subjects, because the cholesterol reductions in the middle density fraction (23 to 49 mg. per 100 ml.) and heaviest fraction (2 to 18 mg. per 100 ml., in 3 of the 4) exceeded the increase (0 to 23 mg. per 100 ml.) in the lightest fraction. The serum cholesterol rose slightly in one subject, because the cholesterol increase (12 mg. per 100 ml.) in the lightest fraction exceeded the slight reductions in the two heavier fractions (total 3 mg. per 100 ml.). Phospholipid changes were parallel.

The disparate concentration changes which occur in the 3 major lipoproteins and their lipids under the above conditions verify and extend Gofman's observations, and underscore the limited utility of the whole serum cholesterol as a guide to dietary therapy. The high correlation of serum triglyceride and triglyceride-rich lipoproteins (S_f 12 to 400) with coronary disease, the dependence of serum triglyceride on the dietary carbohydrate, and the chemical similarity of lipoproteins of density 1.019 and atheroma, emphasize the need for clarifying the role of carbohydrates, as well as fat, in lipoprotein metabolism.

23. Anisotropic lipids and urinary cholesterol excretion

DAVID E. COMINGS, M.D. *Chicago, Ill.*

Introduced by Daniel S. Kushner, M.D.

Frequent observation of anisotropic lipid bodies in the urinary sediment of normal individuals prompted studies of their significance in normal subjects and in patients with various types of renal disease.

Quantitative counts of urinary antisotropic lipid bodies were correlated with urinary cholesterol excretion, protein excretion, serum lipids, and other biochemical variables.

Two types of anisotropic lipid bodies exist in the urine. The true type is found in the urine of patients with the nephrotic syndrome and renal disease with tubular degeneration. Under ordinary light they are small, 1.5 to 8.5 μ in diameter, perfectly spherical, and have no central structure. In polarized light, under low magnification (×100), they appear square, brightly anisotropic, and present a barely discernible, perfectly symmetrical, black Maltese cross. The pseudo type is found in the urine of both normal persons and patients with renal disease. They are larger, 8.5 to 27.0 μ, irregularly spherical, and have a central nucleus-like structure, or fine, crossing furrows. In polarized light, under low magnification, they are spherical, anisotropic bodies with a readily discernible but asymmetric black Maltese cross.

The pseudoanisotropic lipid bodies were present in the urine of almost all subjects. They may arise in part as contaminants from sources such as starch-derivative powder and surgical lubricant and are of importance chiefly in that they may be confused with true anisotropic lipids.

No consistent correlation was observed between the number of true lipid bodies and levels of urinary cholesterol excretion, urinary protein excretion, or serum lipids, but some correlation did exist with the clinical severity of the nephrotic syndrome.

True anisotropic lipids were also found in scrapings of many normal and pathologic nonrenal tissues.

Utilizing a microanalytical technique, the urinary cholesterol excretion for 6 normal control subjects was 547 ± 184 mg. per 24 hours; for 21 nephrotic patients, 487 ± 226 mg. per 24 hours; for 29 nonnephrotic patients with true anisotropic lipids in the urine, 402 ± 310 mg. per 24 hours; and for 25 patients with no true lipid bodies in the urine, 256 ± 160 mg. per 24 hours. There was no consistent correlation between the level of urinary cholesterol excretion and that of urinary protein or serum lipid concentration.

Evidence is presented for the postulate that true anisotropic lipid bodies are a product of cellular reaction and not the result of imbibition of urinary cholesterol by the tubular cells.

24. Hemodynamic effects of a sodium-retaining thiazide derivative: Preliminary observations

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SRG-95213, which is related to the benzothiadiazine diuretics but devoid of the benzenoid sulfamyl group, has been shown to lower blood pressure and yet cause sodium retention (Science 133: 2067, 1961).

Hemodynamic studies in 6 unanesthetized dogs show that this compound, injected intravenously in doses of 1 to 4 mg. per kilogram, is a direct arterial dilator since it causes a fall in blood pressure of 10 to 35 mm. Hg within 14 seconds, with an elevation in right atrial pressure of approximately 0.6 cm. of water and, as indicated by an electromagnetic flowmeter, an increase in stroke volume and cardiac output. The depressor response was potentiated by the prior administration of pentolinium. Chlorothiazide in doses of 5 to 10 mg. per kilogram had no detectable hemodynamic effects.

Oral doses of 200 to 300 mg. per day reduced the blood pressure in 7/16 of the hypertensive patients, the mean for the entire group being 13/9 mm. Hg. There was sodium retention and an average increase in weight of 3.8 pounds. The potential hazard of salt and water retention could be overcome by the simultaneous administration of chlorothiazide, 1 Gm. per day, and this produced an additional hypotensive response in 2/3 of the patients tested.

Present evidence suggests that, although this drug produces an effect on the blood pressure similar to that of chlorothiazide, it does not utilize the same mechanism of action. Its therapeutic usefulness will probably be determined by its ability to enhance the antihypertensive effect of the thiazide diuretics.

25. A new antihypertensive with few side effects*

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With the assistance of T. Fegen, B.S., J. Anslovar, B.S., W. Taylor, B.S., and P. Walters, R.N.

This agent has been subjected to tests in animals that demonstrate that it is a depressor and an antihypertensive. The action involves depletion of tissue catecholamine stores and direct vasodilation (Pharmacologist, vol. 3, No. 2, Fall, 1961).

Clinical trials were begun with the view that this might be a weaker, shorter-acting guanethedine-like agent, laden with side effects of severe sympathoplegia (stuffy nose, diarrhea, postural and exercise hypotension, etc.). Experience to date extends over nearly 6 months in 27 patients selected from the Hypertensive Clinic. In repeated oral doses the agent is effectively antihypertensive in nearly all patients, and has few side effects, the latter being attributable to excessive dosage, which causes tingling and a sense of warmth in the neck and extremities and occasional nausea. Its major disadvantage seems to be its relatively short action (roughly 4 to 6 hours). Studies have also been done of arterial pressure and pressor responses to norepinephrine and angiotensin when injected intravenously in 6 patients on 9 occasions (0.5 to 2.0 mg. per kilogram). These demonstrate in most a transient increase followed by a persistent decrease in pressure, little change or a decrease in norepinephrine responsiveness, and impairment of angiotensin responses in 2 of the tests; these observations are being extended.

Studies of its effects on renal—in 2 tests it is not specifically a renal vasodilator—and cardiac hemodynamics are in progress. At this stage, assuming duration of action of oral doses can be prolonged, as seems assured in preliminary tests, this drug gives promise as an effective antihypertensive agent with modest side effects and no serious problems of tolerance.

Failure to enhance norepinephrine response in patients and absence of sympathoplegic side effects indicate that the mechanism of action differs significantly from that of guanethedine, bretylium, and the ganglioplegics and may be primarily on arterial reactivity.

26. A quantitative method for measuring the cortisol-binding activity of corticosteroid-binding globulin in human plasma

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The existence of a specific binding protein in human plasma with a high affinity for corticosteroid hormones was reported to this society in 1956 (J. LAB. & CLIN. MED. 48: 799, 1956). Reports from this and other laboratories have established that the corticosteroid-binding globulin, CBG, is an alpha glycoprotein and that it largely determines the physical state of circulating adrenocortical hormones. Indirect semiquantitative methods have demonstrated alterations of CBG activity in pregnant women, estrogen-treated patients, and in certain patients with dysproteinemia.

*Tertiary octyl guanidine HCl, BP 1,184, Abbott Laboratories, North Chicago, Ill.

Based on the observations that CBG can be selectively inactivated by heating plasma at 60° C., a quantitative method has been developed for measuring the concentration of CBG-binding sites for cortisol, utilizing double equilibrium dialysis. Two dialysis bags are prepared, one containing 3 to 5 ml. of the plasma which has been heated for 20 minutes at 60° C., the other containing unheated plasma. Both dialysis bags are added to 8 volumes of phosphosaline buffer containing 1 μg of cortisol-4-C^{14} per milliliter of plasma. With this cortisol concentration, equilibrium is reached after 48 hours of continuous agitation at 4° C. and virtually all CBG-binding sites are occupied by cortisol. The difference between the concentrations of C^{14} in the two bags at the end of dialysis is attributable to cortisol-4-C^{14} bound to CBG. Knowing the specific activity of cortisol in the system, CBG-binding capacity can be calculated in terms of micrograms of cortisol per 100 ml. of plasma. All results are corrected for dilution of plasma proteins which occurs during dialysis.

The CBG-binding capacity of 31 plasma samples has been measured. Ten normal adult individuals had a CBG-binding capacity of 17.5 μg per milliliter (S.D. ± 4.2). The CBG cortisol-binding capacity was elevated to between 28 and 66 μg per 100 ml. in plasma from 9 pregnant women. A man receiving 5 mg. per day of diethylstilbestrol had a CBG capacity of 51 μg per 100 ml.

One patient with untreated adrenogenital syndrome had increased CBG-binding capacity, whereas a second patient under treatment had normal CBG cortisol-binding capacity.

27. Comparative effects of lethal amounts of endotoxin on platelets, serotonin, and histamine concentrations in tolerant and nontolerant rabbits

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Introduced by C. J. Watson, M.D.

Recent studies have emphasized the importance of humoral agents in shock due to bacterial endotoxin. Increased concentrations of platelet-free plasma serotonin in rabbits have been shown to be related to lethality, and an increase in plasma histamine has been reported following endotoxin injection in the dog.

To study the relation of serotonin and histamine release to mortality, mature New Zealand white rabbits were made tolerant to a lethal dose of endotoxin by repeated intravenous injections of sublethal amounts of purified *Escherichia coli* endotoxin. A lethal amount was given subsequently, and changes in platelet counts, serum and plasma serotonin, and whole blood and plasma histamine concentrations were determined serially for 2 hours. Serotonin and histamine concentrations were determined by fluorimetry, using published techniques. Control animals consisted of those not previously given endotoxin before a lethal dose, and those given only saline.

Platelet counts increased 15 seconds after saline administration, then stabilized. Fifteen seconds after endotoxin was given to nontolerant animals an abrupt decrease to 59 per cent of the mean control count occurred, then fell to 16 per cent after 5 minutes. In tolerant animals the count fell to 29 per cent of control levels in 15 seconds and to 12 per cent after 5 minutes. During the subsequent 2 hours platelet counts rose, but did not return to control levels. Decreases of serum serotonin and blood histamine were similar to those of platelets after endotoxin administration. The mean concentrations reached about 10 per cent of control levels after 5 minutes, then increased slowly in

both nontolerant and tolerant animals. A significant increase in the concentrations of plasma serotonin and histamine was found 15 seconds after endotoxin was given, but after 5 minutes and subsequently up to 2 hours, no significant increase in the concentration of either amine was found in tolerant or nontolerant animals. In the period immediately after endotoxin, the magnitude of plasma serotonin increase and, to a lesser extent, of plasma histamine increase was smaller in tolerant than in nontolerant animals, suggesting that each amine may be better metabolized in tolerant animals, possibly by stimulation of amine oxidase systems.

28. The diuretic action of progesterone

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An aldosterone-blocking renal tubular action of progesterone has been described in normal subjects, in patients with Addison's disease treated with aldosterone or DOC, and in a few cases of edematous patients with cirrhosis or congestive heart failure. To evaluate this potentially clinically useful diuretic action of progesterone, an experiment was performed using 12 edematous cirrhotic patients in whom the action of progesterone was compared with that of aldactone, a known effective aldosterone-blocking agent, each in combination with a mercurial diuretic agent (Thiomerin).

Using a crossover design experiment, the effect of 200 mg. per day of intramuscularly administered progesterone in oil was compared with that of 400 mg. per day of orally administered aldactone. Each was administered for 1 week, on the last 4 days of which 1 ml. of Thiomerin intramuscularly was administered. The patients were on ad libitum water intake, and Na intake was restricted to 20 mEq. per day. Preliminary urinary excretion studies excluded subjects who were refractory to Thiomerin.

On progesterone-plus-Thiomerin and aldactone-plus-Thiomerin treatments the respective mean daily weight losses were 1.2 and 0.8 pounds, and the mean daily urinary excretions (corrected to 1.73 M^2 S.A.) were as follows, respectively: Na, 111 and 106 mEq.; Cl, 116 and 109 mEq.; K, 61 and 53 mEq.; nitrogen, 8.9 and 7.6 Gm.; and volumes, 1,938 and 1,850 ml. Analysis of variance revealed no statistically significant differences between the treatments as to mean daily urinary volumes, urinary excretions of Na and Cl, and mean weight losses. However, the progesterone-plus-Thiomerin treatment resulted in statistically significantly greater excretions of K ($p < 0.03$) and of nitrogen ($p < 0.001$) attributable to the known catabolic effect of progesterone. This small increase of K excretion (8 mEq. per day) does not constitute a K depletion hazard. Neither treatment significantly altered serum electrolyte concentrations or creatinine clearances.

It may be concluded that 200 mg. of progesterone has an aldosterone-blocking diuretic action on the renal tubule not significantly different from that of 400 mg. of aldactone, and that, in addition, it has a small, clinically unimportant catabolic effect.

29. Absence of an enzyme inhibitor in hereditary angioneurotic edema

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Introduced by Irvine H. Page, M.D.

The present study demonstrates the presence of an alteration in the serum complement system in patients with heredity angioneurotic edema. The serum of these patients lacked the normal inhibitory activity directed against the esterase derived from preparations

of the first component of complement (C'1-esterase). Normal human serum contains 5.8 units of inhibitor of C'1-esterase per milliliter (1 S.D. = 1.8 U.). The serum of each of 4 persons with hereditary angioneurotic edema in 2 families was devoid of detectable inhibitor. Those clinically unafflicted had normal titers, with the exception of one patient's children and 2 other young people. Since age at onset of symptoms is variable, they may develop the disease later. Thus far, patients with nonhereditary chronic or acute angioneurotic edema have been found to have normal or increased amounts of this inhibitor in their sera.

The initial patient in this study had no hemostatic abnormality. Clots formed of her blood remained unlysed after they remained 7 days at 37° C. Blood count and urine were unremarkable. Though her serum lacked inhibitor of C'1-esterase, it contained amounts of plasmin inhibitor and trypsin inhibitor comparable to that found in normal sera. It did not contain activity attributable to C'1-esterase. Upon incubation of patient's serum with normal serum, the inhibitor of C'1-esterase in normal serum remained intact. Thus, we could show no active process at work in the patient's serum destroying this inhibitor. These same observations were repeated during an attack of edema, after its conclusion, and after a symptom-free period of 6 months. The other patients whose serum lacked inhibitor of C'1-esterase had antiplasmin and antitrypsin titers similar to those in normal sera.

These experiments suggest that an inherited abnormality related to the function of the complement system may play a part in hereditary angioneurotic edema.

30. Basic mechanisms of the Schilling test

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Since no detailed knowledge is available as to the basic mechanism of the urinary excretion test, an investigation of this problem was undertaken. Following the oral ingestion of small test doses of radiocyanocobalamin, radioactivity measurements were performed on samples of plasma, ultrafiltrate of plasma, and urine. The endogenous creatinine clearance was used to estimate the glomerular filtration rate. The results indicate that ultrafiltrable radioactivity is present in the plasma in measurable amounts only when the large parenteral dose of "cold" vitamin B_{12} is administered. There was an almost perfect correlation between the urinary radioactivity and the filtered load of radioactivity in the kidneys. Thus, the 2 main factors governing the urinary excretion of radiocyanocobalamin are the amount of ultrafiltrable radioactivity in the plasma and the glomerular filtration rate for this radioactivity.

In vitro vitamin B_{12}–binding studies on plasma obtained before and after the loading injection of "cold" vitamin B_{12} showed that up to 4 hours from the time of the injection plasma had no more binding power for cyanocobalamin than did a solution of normal saline. Starting with the 8 hour plasma sample there was a gradual restoration of the binding capacity for vitamin B_{12}, but even 24 hours after the injection it was not back to the preinjection level. Thus these studies indicated a "saturation" effect of the parenteral cyanocobalamin.

In vitro experiments on plasma showed that an exchange takes place between the protein-bound radiocyanocobalamin absorbed from the intestine and added "cold" vitamin B_{12}. Presumably, a similar exchange occurs in vivo.

Studies were made of the ultrafiltrable radiocyanocobalamin in the plasma before and immediately after the intravenous loading injection in order to elucidate whether the vitamin is absorbed into the blood in a "bound" or "free" form. The results indicate

that 5 and 10 minutes after the injection the major portion of the increment in the plasma radioactivity above the preinjection level was present in a nonultrafiltrable form while as much as one-third was completely ultrafiltrable. It is therefore possible that vitamin B_{12} is discharged into the blood stream in both a "bound" and a "free" form.

31. The effect of hemorrhagic shock on glucose uptake by rat diaphragms

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The marked metabolic alterations that occur with hemorrhagic shock have been ascribed to tissue anoxia occasioned by the circulatory disturbances of hypovolemia. Others have explained the metabolic changes as secondary to the known humoral changes produced by hypovolemia. In tissue, anoxia is known to cause an increased uptake of glucose, whereas epinephrine causes a decreased uptake. The present work was undertaken to explore the alteration in carbohydrate metabolism during hemorrhagic shock in rats when both tissue anoxia and an altered humoral state are present.

Well fed, anesthetized, male, albino Wistar rats weighing 175 to 225 grams were subjected to hemorrhagic shock by an adaptation of the Wiggers canine technique. After a volume of blood equal to 10 per cent of body weight was returned to the shocked rat, both it and a nonbled anesthetized control rat were sacrificed. The hemidiaphragms were incubated for 1 hour in bicarbonate buffer containing 140 mg. glucose per 100 ml. Determinations were made of the following: blood glucose at intervals during the shock procedure; glucose and lactic acid in the incubation medium; and glycogen content of the liver and diaphragm.

The results demonstrated a significantly greater glucose uptake by the diaphragms from the rats subjected to hemorrhagic shock (3.00 μg per milligram tissue per hour—shock; 1.95 μg per milligram per hour—controls; $p < 0.001$). Lactic acid production was approximately the same in both groups (2.12 μg lactate per milligram tissue per hour). At the time of sacrifice, hepatic glycogen was severely depleted in the shocked animals (0.219 Gm. glucose equivalent per 100 Gm. tissue—shock; 3.987 Gm. per 100 Gm.—controls; $p < 0.001$). The glycogen content of the incubated diaphragms was slightly lower in the shocked rats (0.436 Gm. per 100 Gm.—shock; 0.627 Gm. per 100 Gm.—controls). The blood glucose rose, then fell during shock, but was rarely below the control level when the rats were sacrificed.

These results suggest that, during hemorrhagic shock in rats, severe depletion of intracellular energy leads to an increased muscle uptake of glucose despite the concomitant altered humoral state which ordinarily would inhibit glucose uptake. Discussion will consider the therapeutic implications of these results, particularly with regard to "irreversible shock."

32. Hemodynamic responses to administration of acetyl strophanthidin before and after ganglionic blockade in normal dogs and in dogs treated with reserpine

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These experiments were done to see if ganglionic blockade or treatment with reserpine alters the hemodynamic responses to intravenous injection of acetyl strophanthidin. Observations were made on 6 control dogs and 5 dogs which had received 0.05 or 0.1

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mg. per kilogram of reserpine intraperitoneally daily for 5 days. The pressor response to tyramine, 0.1 mg. per kilogram, was almost abolished by these doses of reserpine. Cardiac output (dye), right atrial and systemic arterial pressures, and heart rate were measured before and after injection of acetyl strophanthidin, 0.05 mg. per kilogram, in intact dogs anesthetized with chloralose and urethane. Acetyl strophanthidin was injected twice in each experiment, once before and once after ganglionic blockade with trimethidinium methosulfate, 2 mg. per kilogram.

Increases in arterial pressure, peripheral resistance, and left ventricular minute work, and decreases in cardiac output and heart rate were seen within 1 to 3 minutes after each injection. Changes in right atrial pressure were variable. Following ganglionic blockade the responses of blood pressure, peripheral resistance, and ventricular work were augmented, but the responses of cardiac output and heart rate were not altered. Responses to acetyl strophanthidin were qualitatively similar in control animals and those treated with reserpine.

The greater increase in ventricular work after trimethidinium may result from inhibition of the negative inotropic effect of the vagal stimulating action of acetyl strophanthidin. The increased vasoconstriction after ganglionic blockade is less readily explained, but it is known that reduced ganglionic transmission enhances the effect of a variety of agents which constrict peripheral vessels. Failure of trimethidinium to alter the negative chronotropic response suggests that the direct cardiac action of acetyl strophanthidin may be at least as effective as its vagal stimulating action in reducing heart rate. The data are insufficient to permit quantitative comparison of responses to acetyl strophanthidin in control and reserpine-treated animals. The gross similarity of the responses in these few dogs suggests, however, that the interaction between endogenous catecholamines and acetyl strophanthidin, if it exists, is not a direct one.

33. The anabolic influence of aminopyrine

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Aminopyrine is a highly effective antipyretic, analgesic, and anti-inflammatory drug which has enjoyed varying popularity as a therapeutic agent since its introduction over 75 years ago. Aside from these general properties, however, little information is available concerning its metabolic effects or mode of action.

When administered in usual therapeutic doses (0.3 to 0.6 Gm. every 6 hours) to 3 normal men under carefully controlled conditions, aminopyrine consistently induced a decrease in total urinary nitrogen of 1.5 to 1.9 Gm. daily. The subjects were afebrile throughout the study and there was no lowering of body temperature with aminopyrine. In each instance the positive nitrogen balance was accompanied by a decrease in urinary creatine and inorganic phosphorus. Blood urea nitrogen did not rise during treatment, and possibly diminished slightly in one subject. Urinary creatinine also was unaltered by aminopyrine. The decrease in urinary nitrogen, creatine, and phosphorus occurred within the first 2 days of treatment, and there was a prompt return to base-line values after discontinuance of aminopyrine. Acetylsalicylic acid, which has similar analgesic-antipyretic properties, did not induce nitrogen retention.

In one subject aminopyrine administered during the course of the expected catabolic effect produced by progesterone 50 mg. intramuscularly daily resulted in decreased nitrogen excretion of 1.6 Gm., which is the same magnitude as the effect obtained with aminopyrine alone. The decrease in urinary nitrogen occurred without lowering of the basal body temperature which had been elevated by progesterone.

It is apparent that aminopyrine is anabolic and that this effect is not dependent upon lowering of body temperature. The anabolic effect of aminopyrine with the doses employed averaged 30 mg. of nitrogen per kilogram of body weight. Maximal doses of testosterone have been reported to induce nitrogen retention at about the same rate in normal men. This effect of aminopyrine may provide a lead toward the discovery of more effective nonandrogenic anabolic agents. The anabolic effect of aminopyrine may be related to its therapeutic efficacy, but certainly this property is not inherent in all antipyretic agents.

34. The nature of the acid-base disturbance in salicylate intoxication

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Introduced by W. H. Hall, M.D.

Respiratory alkalosis and a following metabolic acidosis are the changes in the blood acid-base balance known to occur during the course of salicylate intoxication. The factors responsible for the transition of the respiratory alkalosis to the metabolic acidosis have not been clearly defined and are the subject of this experimental study.

Acute intoxication was produced in dogs by the intravenous injection of sodium salicylate. Serial determinations of arterial blood pH, CO_2 content, electrolytes, and lactic and pyruvic acids were performed. The immediate response was severe, continuous hyperventilation resulting in respiratory alkalosis. The persistence of the reduced pCO_2 was associated with the development of a progressive bicarbonate deficit, severe enough to result in metabolic acidosis. This bicarbonate deficit was associated with a rise in the concentration of lactic and pyruvic acids which accounted for the major fraction of this deficit.

In a group of animals, the reduction in blood pCO_2 due to hyperventilation was prevented by breathing 4 per cent CO_2 in air. The bicarbonate deficit and the rise in lactic and pyruvic acids were absent. In an additional series of experiments, the animals were intoxicated with a similar dose of sodium salicylate under conditions of deep anesthesia and complete muscle paralysis. These animals were mechanically hyperventilated using room air and 4 per cent CO_2 in air, respectively, as the respiratory mixture. Under these conditions the changes leading to the development of the metabolic acidosis, i.e., the progressive bicarbonate deficit and the rise in lactic and pyruvic acid concentrations, were observed only during the experimental periods in which pCO_2 was reduced.

It is therefore concluded that the metabolic acidosis in salicylate intoxication develops as a consequence of changes precipitated by the initial and prolonged reduction in pCO_2.

35. The in vivo genesis of the coagulation defect complicating pathologic plasma proteolytic ("fibrinolytic") states: Study of an iatrogenically induced lesion

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In vitro evidence has been presented that the coagulation defect developing during pathologic plasma proteolytic states is related to fibrinogen proteolysis. Proteolysis of fibrinogen by plasmin results in formation of a large molecular weight fibrinogen fragment (5.27 S_{20w})

which, circulating in plasma, interferes with the clotting of fibrin monomer. We have termed this anomaly "defective fibrin polymerization" (J. LAB. & CLIN. MED. 56: 813, 1960).

Further in vitro experiment, using I^{131}-labeled proteolysis products, has demonstrated that the extent of proteolysis product incorporation into clots formed from plasma containing them reflected the severity of the induced coagulation defect. Fibrin digests, as well as fibrinogen digests, also inhibit fibrin polymerization, a finding relevant to the "spontaneously developing" fibrinolytic state, where lysis of intravascular fibrin deposits may enhance the severity of the coagulation defect.

In the present study the over-all quantitative significance of circulating fibrinogen proteolysis products, with respect to the development of a coagulation defect, was investigated in patients developing pathologic plasma proteolytic states during streptokinase therapy. Serial assays for coagulation and plasminogen-plasmin system components were made during the developing phase of the coagulation defect in 22 patients. A coagulation defect, evinced by a rise in the thrombin and one-stage prothrombin times, developed even though plasma fibrinogen was reduced to low normal levels only. However, fibrinogen proteolysis product formation, calculated from the rate of plasma fibrinogen fall, was linearly correlated with the degree of coagulation defect, measured by prolongation of the thrombin time. In vitro experiment indicated that in vivo fibrinogen proteolysis product formation was sufficient to account for the total over-all coagulation defect observed. Observations during the recovery phase showed that the 50 per cent plasma clearance rate of the abnormal fibrinogen fragment averaged 9.5 hours.

These observations establish the importance of defective fibrin polymerization in the genesis of the coagulation defect occurring in pathologic fibrinolytic states.

36. The effect of insulin in myasthenia gravis

MARCEL FRENKEL, M.D. *Chicago, Ill.*

Introduced by Theodore N. Pullman, M.D.

The intravenous infusion of insulin under standard conditions induced marked improvement in 5 patients with myasthenia gravis.

Fifty units of regular insulin and 1,000 ml. of 10 per cent dextrose containing 40 mEq. of potassium chloride were given in 1 hour. Control infusions without insulin were also administered. Strength was measured by work tolerance tests, weight lifting, and ergographic recordings. Performances were recorded by cinematography and photographs; they also were judged by independent observers.

A dramatic increase in strength was demonstrable in all cases within 45 minutes of the start of the infusion and maintained for as long as 2 hours. In one mild case, improvement was obtained without associated use of an anticholinesterase. In the other cases, the effect was observed when treatment was started at the peak action of Prostigmine, but not when all Prostigmine influence had been dissipated. Improvement was also seen when the insulin-containing solution was given well past the peak effect and 10 hours after the last dose of the anticholinesterase drug, Mytelase. Hyperglycemia or normal blood glucose concentrations were necessary for maintaining the enhanced strength. Benefit was rapidly dispelled when hypoglycemia was allowed to occur, and strength was restored when glucose was given orally. Intravenous glucose alone did not affect the strength, but slight improvement was observed following potassium chloride infusion with or without glucose.

None of the patients was frankly diabetic. Glucose tolerance was mildly impaired in 2 patients. Serum potassium, sodium, and chloride concentrations were normal in all patients, both before and during the treatment periods. Serum inorganic phosphate was expectedly lowered by the insulin, potassium chloride, and glucose infusions.

It is suggested that the effect of insulin in these cases may be mediated by its known action in altering membrane permeability and the rate of potassium transfer into cells, thereby affecting neuromuscular excitability.

37. The thyroid cytotoxic factor

I. J. FORBES, M.R.A.C.P., I. M. ROITT, PH.D., D. DONIACH, M.D., and I. F. L. SOLOMON, M.D. *Ann Arbor, Mich.*

Introduced by W. H. Beierwaltes, M.D.

The finding by Pulvertaft and associates in 1959, that the serum of patients with Hashimoto's disease would kill human thyroid cells in tissue culture, has made possible study of the action of serum factors on living cells.

Trypsin-dispersed human thyroid cells were cultured in inverted 0.8 ml. capacity chambers. Medium containing normal serum allowed thyroid epithelial cells to become established on the surface of the cover glass which sealed the chamber, whereas the presence of specific thyroid cytotoxic factor prevented their establishment.

The antibody nature of the cytotoxic factor was established by showing that (1) complement was necessary for its cytolytic action, and (2) it was contained in the gamma globulin fractions of serum obtained by zone electrophoresis on "Pevikon" blocks, sodium sulfate precipitation, and DEAE cellulose column chromatography. The identity of the fractions was confirmed by immunoelectrophoresis. It was shown that the cytotoxic antibody, if present in sufficient concentration, fixed complement with toxic thyroid microsomes. Eighty-three of 85 sera which fixed complement with antigen derived from toxic thyroid tissue were cytotoxic. Forty-five sera which were negative in the complement fixation test were cytotoxic.

Studies using fluorescent antibody technique on cells in culture indicated that the antigen was distributed throughout the cytoplasm, but that the cytolytic action of the antibody was due to combination of the antibody with antigen present on the cell surface.

Analysis of the results of 49 cultures of thyroid tissues showed that cells from glands of Graves' disease were regularly highly sensitive to the cytotoxic antibody, cells derived from toxic nodular goiters were variably sensitive, whereas normal thyroid and nontoxic nodular goiters yielded cells which were slightly sensitive or quite insensitive.

The highest incidence of the factor was found in Hashimoto's disease (96 per cent), but it was present in two thirds of cases of myxedema and thyrotoxicosis. The incidence in other thyroid disease was low except for the close relatives of persons having Hashimoto's struma or Graves' disease (29 per cent). The incidence in the control series was 9 per cent.

38. Effects of angiotensin upon the myocardium and the coronary circulation

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Cincinnati, Ohio

1-L-asparganyl-5-L-valyl angiotensin octapeptide (hypertensin, CIBA) is a potent pressor agent in normal and hypotensive animals and human subjects. The drug is known to constrict small systemic arteries, but its cardiac effects have not been thoroughly studied. In the present study the direct cardiac effects of angiotensin were investigated in the dog heart-lung preparation and its effect upon the coronary circulation was evaluated during constant flow perfusion of the descending branch of the left coronary artery of the dog. Cardiac force was measured by a strain gauge arch sutured to the right ventricle. Coronary flow was measured directly by cannulation of the coronary sinus.

Ten to 40 μg of angiotensin had consistent effects in 10 dog heart-lung preparations. There was an early decrease in cardiac output (Avg. −24.1 per cent), coronary flow (Avg. −30.4 per cent), contractile force (Avg. −4.2 per cent), and blood pressure (Avg. −6.5 per cent). Ten to 15 seconds later there was a significant increase in the cardiac output (Avg. +18.7 per cent), coronary flow (Avg. +23.0 per cent), contractile force (Avg. +15 per cent), and systemic blood pressure (Avg. +5.3 per cent). A significant early rise in right atrial pressures followed by a decrease below control levels was observed. Changes in cardiac rate (Avg. −1.2 per cent) were slight. Similar results were obtained in 4 preparations in which the cardiac rate was controlled.

In 10 dogs, using the method of constant flow perfusion of the descending branch of the coronary artery, 1.0 μg angiotensin produced consistently an immediate increase in coronary vascular resistance (Avg. +55.7 per cent) with a delayed carotid arterial pressure increase (Avg. +3.1 per cent). Concomitant with increased coronary resistance, the contractile force decreased (Avg. −6.6 per cent).

It is concluded that angiotensin has direct cardiac effects with an initial depression of cardiac output, coronary flow, and contractile force followed by an increase in all 3 of these parameters. The initial depression of cardiac output appears to be caused at least partly by an increase in coronary vascular resistance.

39. Bioassay and "transport" of plasma insulin after massive insulin treatment of diabetic ketoacidosis

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A patient with severe diabetic ketoacidosis received 15,000 units of regular insulin in 18 hours. Plasma samples were obtained for insulin bioassay (mouse hypoglycemia) over the succeeding 6 days, during which no further insulin was given. Very great activity (130 milliunits per milliliter) was detected 90 minutes after the last insulin dose, and appreciable activity (12 mu. per milliliter) was still present 2 days later. The state of insulin in these plasma samples was further studied by starch block electrophoresis and separation on a Sephadex column. In the earliest sample, most of the biologic activity had the same mobility as α_1 and α_2 globulin, but in the samples drawn 1 and 2 days later peak biologic activity had shifted to beta and gamma globulin. Sephadex separation indicated that some of the insulin associated with α_2 globulin was protein bound, but an appreciable portion was not bound. The patient's clinical course showed continued effectiveness of the circulating insulin even when it was associated primarily with beta and gamma globulin.

The results in this patient indicate that circulating exogenous insulin may be biologically effective irrespective of the plasma fraction with which the insulin activity is associated.

40. A simple immunoassay for small amounts of insulin

FREDERICK C. GOETZ, M.D., and BERYL GREENBERG, M.S. (By Invitation) *Minneapolis, Minn.*

A simple, rapid, and highly reproducible immunoassay for small amounts of insulin has been devised, based on the globulin precipitation method of Skom and Talmage for detection of insulin-binding antibody. The assay is made possible by the commercial availability of I^{131}-labeled insulin of moderately high specific activity (25 to 30 mc. per milligram). Nearly identical dose-response curves over the range 1 to 10,000 microunits are obtained

with pure beef-pork or pork insulin, crude human or dog insulin, and dog plasma rich in endogenous insulin. The method has been satisfactorily applied to peripheral and pancreatic venous plasma from the dog. Insulin concentration in pancreatic plasma after glucose stimulation is about 5,000 to 10,000 microunits per milliliter; bioassay (mouse hypoglycemia) of the same samples gives essentially identical results.

41. Calcium kinetic studies in hypoparathyroidism

RICHARD GOLDSMITH, M.D., JOHN WULSIN, M.D. (By Invitation), and MILDRED J. WIESTER, B.S. (By Invitation) *Cincinnati, Ohio*

The calcium kinetics for hypoparathyroidism, as observed by an intravenous tracer dose of Ca^{45}-labeled calcium, were measured in dogs. The method was the classical balance technique. The dogs were prepared by total surgical thyroparathyroidectomy. After being allowed to become hypocalcemic and tetanic the animals were maintained on oral dessicated thyroid substance and enough supplemental calcium (in the diet and by infusion) to allow them to be reasonably healthy and active. They were studied while in metabolic cages. Each study extended over a period of approximately 1 month. Included in this report are the results of 5 studies performed on 4 hypoparathyroid animals and 1 control animal.

Observations were made on the stable and labeled calcium content of blood, urine, and stool during the course of each study. The specific activity curves for urine and blood were constructed and appropriate regression lines were calculated. The data described a biphasic exponential curve for each animal. The first phase apparently represented the fractional rate of labeling of the eventual readily exchangeable body calcium pool. The second phase apparently represented the fractional rate of turnover of that pool after saturation with the label had occurred. The hypothyroid dogs demonstrated a slower rate of pool saturation than did the control animal, $k = 0.513$, 0.327, and 0.161 for the former and 0.950 for the latter; the rate of turnover after saturation had occurred was very slow in all 4 animals. The size of the eventual pool of readily exchangeable calcium found at the time of saturation was considerably less for the hypothyroid than for the control animals on a weight basis.

The hyperphosphatemia observed in all the hypothyroid animals was eliminated in 1 animal by probenecid administration. During the normophosphatemic interval, and while yet hypocalcemic, the animal was restudied with a second tracer dose of Ca^{45}. At that time the kinetics observed were more those of the control animal. It is suggested, therefore, that such kinetic studies give information regarding the nonhormonally controlled exchange of bone and extracellular fluid calcium rather than information regarding the parathormone-controlled bone reactions.

42. Energy metabolism in obesity

EDGAR S. GORDON, M.D. *Madison, Wis.*

Obesity has been found to be associated with a number of well-documented metabolic anomalies which may be regarded as supplementary to the old basic proposition that this condition is caused entirely by an excessive caloric intake, too little expenditure of energy, or a combination of the two. These anomalies may be listed as (1) a low fasting and even postprandial respiratory quotient, often, if not usually, below 0.8; (2) a curious and thus far unexplained resistance to the development of ketosis under fasting conditions; (3) resistance to normal metabolic nitrogen loss in response to dietary caloric and protein restriction; and (4) an abnormally high level of free fatty acids in the blood.

This study of pathologically obese and normal control human subjects involves the measurement of fluctuations in blood concentration of free fatty acids, ketones, triglycerides, and glucose throughout a day of fasting. Intravenous injection of C^{14} 1-palmitate, C^{14} 1-glucose, and C^{14} 6-glucose under these conditions has permitted continuous recording of expiratory CO_2 and $C^{14}O_2$ as an index of total oxidation of these compounds. The effects of glucose loading and the intravenous administration of human somatotrophin have also been studied.

The significant findings are as follows: (1) Under fasting conditions, the blood free fatty acid level does not rise as in normal subjects but remains essentially flat, even through several days of complete fasting. (2) Obese subjects show a significantly elevated level of triglycerides as compared with normal subjects. (3) Increased rates of free fatty acid mobilization in response to somatotrophin administration are demonstrable in both obese and normal subjects. (4) Increased rate of oxidation of free fatty acids and production of ketones from somatotrophin injection may occur in obese subjects only. (5) Following glucose loading, glucose oxidation is markedly suppressed in obesity, and palmitate oxidation is very active, or even stimulated, in obese subjects.

Many of these findings are compatible with the hypothesis that a deficiency of circulating somatotrophin occurs in obesity. Some of the findings may be the result rather than the cause of obesity.

43. Thiadiazine treatment of hypertension: A paired experiment with sequentially administered benzydroflumethiazide, potassium chloride, and spironolactone

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Introduced by Ford K. Hick, M.D.

The mechanism of chronic action of thiadiazines in hypertension in unclear. In a paired experiment with 68 nonedematous hypertensive outpatients serving as their own controls, benzydroflumethiazide (BF) decreased the mean pressure of the group 13 per cent ($p < 0.001$). Responsiveness was randomly distributed and bimodal distribution did not occur. Responsiveness was statistically unrelated to age, weight, degree, or duration of hypertension, BUN, or sodium intake, which averaged from 45 to 260 mEq. daily. In balance studies on inpatients with restricted diets, BF produced negligible potassium and often only slight sodium losses, not correlated with pressure responsiveness. Serum potassium and chloride decreased and CO_2-combining power (CO_2) rose despite some rise of BUN. The latter was inadequate to explain the sharp rise in serum uric acid. Additional sodium or potassium chloride reversed these changes. The potassium increased the pressure, sodium did not.

Adding spironolactone to BF therapy lowered only the systolic pressure and repaired the hypokalemic alkalosis, but not the hyperuricemia, despite the production of hyperkalemia and persistence of hypochloremia. The CO_2 decreased unproportionally to the increasing BUN. Hyperuricemia was not increased despite increasing azotemia. Several developed potentially dangerous hyperkalemic acidosis.

We propose that these discordant chemical changes, the hypokalemic alkalosis, and perhaps the hyperuricemia associated with BF therapy, are a result of secondary hyperaldosteronism. These clinically unimportant but statistically significant chemical changes attest to successful homeostatic adaptation to the stimulus of daily repetitive natruresis from BF.

Opposing effects of high and low sodium intake on serum CO_2 and potassium in patients with primary hyperaldosteronism compared with those treated with thiadiazine are noted and the diagnostic usefulness mentioned. Compared to normotensive control subjects untreated hypertensive patients did not have hyperuricemia but did have elevated serum CO_2-combining powers (27.3 ± 0.2* versus 28.2 ± 0.3* mEq. per liter), despite mild azotemia agreeing with other evidence relating increased aldosterone and untreated hypertension. Monthly quantitative urine cultures remained negative despite prolonged hypokalemia. Restriction of sodium and/or addition of potassium with routine thiadiazine treatment of hypertension is unnecessary and perhaps undesirable.

44. Stem cell kinetics

C. W. GURNEY, M.D., and L. G. LAJTHA, M.D. (By Invitation)
Chicago, Ill.

Newly devised techniques permit in vivo investigation of stem cell kinetics of bone marrow. In addition to demonstrating the potential of stem cells to regenerate after injury, these studies support new concepts of stem cell regulation.

Erythropoiesis can be eliminated in mice by hypertransfusion, and quantitative erythropoietic responses follow administration of graded doses of erythropoietin. In hypertransfused mice irradiated immediately before injection of erythropoietin, the erythropoietic response was impaired; over a wide range the log of the erythropoietic response, as measured by per cent erythrocyte incorporation of Fe^{59} administered 48 hours later, decreased in linear fashion with increasing radiation damage.

Since erythropoiesis was almost absent at time of irradiation (reticulocytes less than 0.05 per cent), impairment of response to erythropoietin is presumed to measure damage to the stem cell. It is thus possible, in vivo, to obtain measurements of the proportion of population of mammalian cells which retain their reproductive integrity after being subjected to varying doses over a wide range of irradiation.

By varying the time after irradiation at which mice are challenged with a constant dose of erythropoietin, recovery of the stem cell compartment may be assessed. For example, when erythropoietic stimulus was given immediately post irradiation (150 r), the Fe^{59} incorporation fell to 11 per cent, as compared with 33 per cent in nonirradiated controls. If, however, erythropoietic stimulus was delayed until 24 hours post irradiation, further decrease in the number of viable stem cells available for erythrocyte formation was noted, only 5 per cent of the Fe^{59} being incorporated into newly formed erythrocytes. The number of stem cells available for erythropoiesis then increased slowly, the compartment being only slightly more responsive to erythropoietin 5 days after irradiation than immediately following 150 r. The rate of recovery then suddenly increased and in the next 24 hours returned precipitously to normal.

Patterns, rates, and times of recovery of stem cells following different doses of irradiation are consistent with new concepts which attempt to account for normal maintenance of the stem cell compartment and for regeneration following injury, despite continuous attrition in the form of egress of cells from the stem cell compartment in response to stimuli for differentiation.

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*Standard deviation of mean ($p = 0.02$).

45. Immunization with viable Brucella organisms: Results of a safety test in human volunteers

JAMES W. HALL III, M.D. (By Invitation), WESLEY W. SPINK, M.D., JOANNE FINSTAD, M.S. (By Invitation), and EDMUND MALLET (By Invitation) *Minneapolis, Minn.*

The reservoir of human brucellosis is infected livestock. Bovine brucellosis due to *Brucella abortus* has been partially controlled by immunization with an attenuated living vaccine of abortus (strain 19). Comparable results in large human populations have been reported by Russian investigators in which protection with strain 19 has been successful against the more virulent *Brucella melitensis*. Because human brucellosis caused by *Br. melitensis* constitutes a serious threat to health in many parts of the world a special World Health Organization (WHO) committee requested a safety trial in human volunteers with two viable Brucella vaccines. Vaccine 19A, *Br. abortus* was supplied from Moscow through WHO. A second vaccine, Rev. I, was derived from *Br. melitensis* by Dr. S. S. Elberg of the University of California and showed attenuated virulence in several species of animals. Two groups of 16 healthy men, 21 to 50 years of age, without evidence of previous exposure to brucellosis, were each given a recommended standardized dose of one of the viable vaccines. They were observed for a period up to 6 months during which time serologic and bacteriologic studies were made. These findings were correlated with the appearance of Brucella intradermal hypersensitivity. Four of the 16 recipients of vaccine 19A developed acute brucellosis, 2 of whom were hospitalized. Brucella (19A) was recovered from the blood culture of only 1 of the 16 volunteers. Acute brucellosis occurred in 12 of the 16 receiving Rev. I vaccine, 4 being hospitalized. One developed acute Brucella pyelonephritis. Brucella (Rev. I) organisms were recovered from the blood 2 and 3 weeks following vaccination in 12 of 16 men. All of the 32 individuals had Brucella agglutinins, the Rev. I group having higher titers. All 16 receiving Rev. I demonstrated dermal Brucella hypersensitivity, while 5 of 16 receiving 19A vaccine failed to do so. All 16 individuals receiving Rev. I vaccine were treated with tetracycline and all promptly recovered. Tetracycline also was successfully used in the 4 men with illness due to 19A vaccine. Until further information, with different dose schedules, is forthcoming, neither vaccine can be recommended for unlimited distribution.

46. Factors limiting hydrogen ion excretion in renal disease

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Introduced by George J. Gabuzda, M.D.

This study was done to determine whether the acidosis of renal disease is due predominantly to limitation of hydrogen ion secretion or of available buffer in tubular urine. By measuring hydrogen ion excretion when phosphate buffer was provided, the tubular secretion of hydrogen ion was evaluated separately from the production of buffer (ammonia).

Three patients with chronic renal disease with nitrogen retention and one patient with renal tubular acidosis, manifested by an inability to acidify urine, were studied. Two of the azotemic patients were spontaneously acidotic. The third was given 25 mEq. of hydrochloric acid daily by mouth to produce a progressive systemic acidosis. The patient with renal tubular acidosis was given 80 mEq. of hydrochloric acid daily to induce maximum acid excretion. When net acid excretion (the sum of titratable acid plus ammonia minus bicarbonate) had reached a maximum and acidosis persisted, each subject ingested, daily, 40 mEq. of phosphate in a solution containing the mono- and disodium salts at pH 7.4. This was continued for 9 days.

In the presence of systemic acidosis the azotemic patients excreted urine containing little ammonia and ranging from pH 4.8 to pH 5.2. The administration of phosphate re-

sulted in increased phosphate and titratable acid excretion without changes in urine pH or ammonia. A net increase in hydrogen ion excretion resulted. The plasma pH and bicarbonate concentrations returned to control levels. The patient with renal tubular acidosis, in contrast to the azotemic patients, increased urinary ammonia excretion but failed to acidify the urine below pH 6.2 when given acid. The administration of phosphate buffer resulted in increased urinary phosphate excretion without change in urine pH. The increase in urinary titratable acid was accompanied by an equivalent decrease in ammonia excretion, the metabolic acidosis persisting. Net hydrogen ion excretion remained unchanged.

The subject with renal tubular acidosis demonstrated limitation of ability to secrete hydrogen ion. In contrast, the azotemic patients increased hydrogen ion excretion when phosphate was given. In these patients the predominant factor limiting hydrogen ion excretion was availability of buffer.

47. Myocardial metabolic influence of glucagon and associated contractile and rhythmic responses

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The recent demonstration of phosphorylase activation in the isolated heart following glucagon (Fed. Proc. 20: 85, 1961) establishes after prior failure a direct myocardial effect of this hormone and suggests that it may serve a regulatory role in myocardial function.

Since the cardiac effects of purified glucagon have not been described in the intact animal, 7 anesthetized dogs were given 50 to 70 μg of purified glucagon into the left coronary artery to minimize systemic metabolic influence. The duration and maximum rate of left ventricular isometric pressure rise were used as indices of contractile response. Within 10 seconds an increase in left ventricular contractility occurred, a maximal increment of 80 per cent occurring by 2 minutes without altered ventricular pressures. A transient nodal tachycardia at 30 seconds was followed by a sinus tachycardia and both inotropic and chronotropic responses persisted 30 minutes.

Myocardial metabolic response, followed sequentially by arteriovenous differences, was marked by an immediate increase in glucose extraction, with a decline in extraction of free fatty acids, lactate, and pyruvate, persisting for 2½ minutes. After the major rise in glucose extraction began to decline, pyruvate and lactate extraction increased from 5 to 30 minutes, in what would appear as release from the competitive inhibition of accelerated glucose transport. While this latter phenomenon was associated with initiation of contractile response, its functional relationship remains speculative.

Since most of the hemodynamic and metabolic responses mimic those of epinephrine, the inhibitor dichloroisoproterenol was infused into the left coronary artery prior to glucagon in 5 experiments and was found to largely reduce the contractile response. A substituted propriophenone which sensitizes the myocardium to epinephrine-induced ventricular fibrillation markedly prolongs ventricular repolarization, a pattern reversed by glucagon. In no instances was ventricular fibrillation or ectopic foci observed with large doses of this hormone. This appears to be the only evident difference between glucagon and epinephrine and suggests its possible usefulness as an inotropic substance.

48. Radioisotope renogram as sensitive test of kidney function

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With the assistance of S. A. Cech, B.S.

While the radioisotope renogram was proposed primarily as a means of detecting differences in function between kidneys, the procedure is intrinsically simple and, if reproducibly quantitatable, would be an excellent clinical index of renal excretory function. Previous interpretations have been at best no more than semiquantitative, so that renogram interpretation is still highly subjective (Klapproth, H., Hirakawa, A., and Corcoran, A. C.: J. Urol. [in press]). This report describes a means of quantitative interpretation which has been evaluated in terms of reproducibility and by reference to excretion rate of the isotope carrier (Hipputope) in 40 normal subjects and in 72 patients with hypertensive or renal disease of varying severity. The index is based on measurement of the initial peak (A), second peak (B), and terminal (15 minutes) level (C), from background base line and calculation of an index as $(B-A)^2 + (B-C)^2/B^2$ (normal 0.61 ± 0.06, 20 minute excretion rate in 36 cases 64.7 ± 6 per cent of injected I^{131}). In hypertensive subjects with respective BUN levels of 0 to 14, 15 to 24, 25 to 44, and 44 or more milligrams per 100 ml., renogram indices were 0.41 ± 0.14, 0.29 ± 0.1, 0.22, and 0.06, with excretion rates of 43 ± 15, 37 ± 17 19 ± and 3 per cent, the values listed being means and standard deviations. Intravenous pyelograms were done in 63 hypertensive patients and 1 apparently "normal" subject with latent pyelonephritis; 49 were reported as normal in all respects. The renogram is an accurate, highly sensitive screening index of renal impairment.

49. The influence of position on sodium space

C. J. HLAD, JR., M.S. (By Invitation), E. R. HUFFMAN, M.D. (By Invitation), S. GUGGENHEIM, B.S. (By Invitation), and H. ELRICK, M.D. *Denver, Colo.*
Introduced by R. J. Glaser, M.D.

Previous studies in man indicate that plasma volume contracts following a change from the recumbent to the standing position. In an earlier study, the authors measured the volume of distribution of sodium (sodium space) utilizing a new continuous infusion technique and Na^{22}. This method has been applied to demonstrate the influence of position on sodium space.

In 11 normal subjects, sodium space was measured during quiet standing. The mean sodium space was 16.7 per cent of body weight with a standard deviation of 1.8 per cent. This value is 26 per cent below the mean sodium space in the supine position and this difference is highly significant ($p < 0.001$). This difference is much greater than changes in plasma volume with standing.

In 8 subjects, sodium space was measured during several changes in position. Although the magnitude of response was variable, it was consistently observed that sodium space decreased upon standing and increased in the supine position.

In 5 subjects, a single dose of Na^{22} was injected intravenously 12 hours prior to the study. During the study, blood samples were obtained in the standing and supine positions and no change in sodium concentration was observed. In 5 other subjects, a single dose of Na^{22} was injected intravenously with the subject in a standing position. In this position, blood samples were obtained for at least 30 minutes. The plasma concentration of Na^{22} decreased over this time interval at a rate much slower than that associated with extracellular distribution of Na^{22} in subjects in the supine position. These same subjects

were then placed in a supine position and plasma measurements continued. In all cases, an abrupt drop in plasma concentration occurred.

These findings indicate that sodium space is markedly reduced in the standing position. This reduction in sodium space does not necessarily indicate a reduction in total extracellular fluid volume. A small part of the reduction in sodium space is almost certainly due to a reduced plasma volume. However, it is postulated that the major portion of this reduction is the result of decreased sodium transfer secondary to reduced blood flow in certain areas.

50. The relation between fingerprint patterns and Wilson's disease

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(By Invitation) *Iowa City, Iowa*

Wilson's disease is known to be transmitted as a Mendelian recessive. It occurs with greatest frequency in the offspring of consanguineous marriages. This implies that both parents carry the trait. Previous attempts to detect this trait by measuring amino acid secretion, concentrations of copper and ceruloplasmin in the serum, and transportation of radioactive labeled copper have been partially but not completely successful.

Fingerprints are an inheritable characteristic of all persons. They are subject to precise classification. They are transmitted as Mendelian dominants. We investigated the possibility that there might be some correlation between the type of fingerprint and the presence of Wilson's disease. In this preliminary report, we present data from 20 patients with Wilson's disease compared with data from a control group of 200 normal persons. These data describe a highly significant increase in the incidence of whorls on the thumb, index, and ring fingers of patients with Wilson's disease. Preliminary observations of the relatives of patients with Wilson's disease will be discussed.

51. Changes in glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activity in rat adipose tissue following carbohydrate ingestion

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Introduced by William S. Jordan, Jr., M.D.

The ability of adipose tissue to convert carbohydrate to fatty acids for storage in fat depots appears to be a major factor in the maintenance of normal blood sugar levels when an animal is challenged with a large carbohydrate load. This lipogenetic response of adipose tissue is limited by the amounts of glucose-6-phosphate (G-6-P) dehydrogenase and 6-phosphogluconic (6-P-G) dehydrogenase available for the generation of reduced triphosphopyrimidine nucleotide (TPNH).

We have studied the mechanism by which G-6-P and 6-P-G dehydrogenase activity in rat adipose tissue rises in response to a large carbohydrate load, with the following findings: (1) G-6-P and 6-P-G dehydrogenase activity in adipose tissue rose 30 per cent and 50 per cent, respectively, 1 hour after rats were given 4 Gm. of glucose per kilogram by stomach tube. The activity of these enzymes fell to fasting levels in 2 hours. (2) The activity of these enzymes in liver did not rise during a 4 hour period following glucose loading. (3) G-6-P and G-P-G dehydrogenase activity in adipose tissue from rats trained to eat their entire day's ration in 2 hours rose approximately tenfold by the end of the 2 hour feeding period. Adipose tissue homogenates and serum from fed and trained rats did not increase

the activity of these enzymes in homogenates from fasting animals. Excesses of TPN, TPNH incubation with insulin, and glucose did not increase the activity of these enzymes in homogenates from fasted rats.

These studies suggest that increases in G-6-P and 6-P-G dehydrogenase activity in adipose tissue following ingestion of carbohydrate are the result of synthesis of these enzymes rather than activation of inactive preformed enzymes. This rapid response of adipose tissue to a carbohydrate load, and the adaptive changes which occur with repeated challenge raise many questions concerning the mechanisms of glucose intolerance in starvation and the improvement in glucose tolerance in diabetes with onset in maturity following weight reduction.

52. The intestinal brush border as a digestive surface: Localization of aminopeptidase and phosphatase in the mucosal epithelial cell

JOHN H. HOLT, M.D., and DAVID MILLER, M.D. *St. Louis, Mo.*

Introduced by Albert B. Eisenstein, M.D.

Hydrolysis of disaccharides and sugar phosphate esters in the intestine has been shown to be a function of intact mucosal epithelial cells and is not due to extracellular action of enzymes present in succus entericus. Following localization of invertase and maltase in the brush border portion of intestinal epithelial cells, it was proposed that the striated border of mucosal epithelium constitutes a digestive surface for carbohydrates. A method previously described (Anal. Biochem. 2: 284, 1961) for isolating brush borders has now been applied to subcellular localization in mucosal epithelium of aminopeptidase and acid and alkaline phosphatases.

Hamster intestinal mucosa was homogenized in dilute ethylenediaminetetraacetic acid and brush borders were isolated by low speed centrifugation. In order to determine whether a given enzyme was localized in the brush border or some other part of the cell, enzyme activity and protein content of the whole homogenate and of the brush border suspension were determined. Aminopeptidase was assayed by hydrolysis of L-leucyl-β-naphthylamide and both acid and alkaline phosphatases were determined by hydrolysis of p-nitrophenyl phosphate at the appropriate pH. With both aminopeptidase and alkaline phosphatase approximately 75 per cent of the activity of the whole homogenate was recovered in a brush border preparation which contained only 10 per cent of the total protein of the original homogenate. In the case of acid phosphatase only 25 per cent of the total activity present was found in brush borders. These results are consistent with published histochemical data obtained using similar substrates.

The predominant localization of peptidase activity in the mucosal brush border suggests that terminal digestion of proteins as well as carbohydrates is a function of this subcellular structure. Similar findings for alkaline phosphatase indicate that the function of this enzyme in intestinal mucosa may be, at least in part, a digestive one. In contrast, intestinal acid phosphatase is more widely distributed in the epithelium and is not specifically localized in the brush border.

53. Acquired resistance to reinfection in experimental ascending pyelonephritis

BETTY W. HUNTER, M.D. (By Invitation), LONA L. SOUDA, B.S. (By Invitation), and JAY P. SANFORD, M.D. *Dallas, Texas*

Acquired resistance to gram-negative infections is of 2 types: the first develops within hours after injection of endotoxin, is not type specific, and persists only a few days; the second is classical immunity associated with circulating antibodies, is type specific, and persists for

weeks. Despite earlier impressions, recent studies demonstrate that both types of resistance are important factors in the pathogenesis of experimental hematogenous pyelonephritis. However, the protection demonstrated against hematogenous pyelonephritis might be the consequence of accelerated clearance of bacteria from blood with lessened renal challenge rather than their elimination from renal parenchyma. These studies define the role of acquired resistance in the genesis of ascending pyelonephritis in which accelerated vascular clearance is not a factor.

Pyelonephritis was produced in rats by injection of *Proteus mirabilis* into the bladder. The strain, used uniformly, produced acute pyelonephritis with gross abscesses, bacterial counts of $> 10^6$ per gram of kidney and, frequently, azotemia. The infection evoked an immune response against proteus O antigen (mean 7 day titer 1:640). Rats were immunized by: (1) production of acute pyelonephritis, then eradication of the infection with antibiotics (chloramphenicol and kanamycin); (2) subcutaneous injection of boiled, formalinized organisms; and (3) passive transfer of rabbit *Pr. mirabilis* antiserum. Following eradication of previous infection, there was significant protection upon rechallenge. Gross abscesses: control, 1.5+; immunized, O. Organisms: $\log^{10}$ per gram kidney, control, 7.9 ± 3.3; immunized, 0.6 ± 1.4 ($p < 0.01$). BUN: control, 60 ± 34 mg. per cent; immunized, 27 ± 2 mg. per cent; ($p < 0.05$). Similar protection was noted of animals immunized with killed organisms. Gross abscesses: control, 1.3+; immunized, O. Organisms: $\log^{10}$ per gram kidney, control, 6.0 ± 3.9; immunized, 1.5 ± 1.8 ($p < 0.01$). BUN: control, 60 mg. per cent; immunized, 28 mg. per cent. Passive immunization failed to protect against challenge. This may indicate either that resistance acquired by infection or antigen represents early nonspecific resistance or tolerance rather than classical immunity, or that insufficient antisera was transferred.

In summary, the acquisition of resistance to ascending proteus pyelonephritis again demonstrates that immunity is an important factor in experimental recurrent pyelonephritis. These data also exclude accelerated clearance of bacteria from blood as the sole mechanism of resistance.

54. Evaluation of a dialysis method for PBI^{131} determination

JOHN J. IMARISIO, M.D. (By Invitation), and
BRUNO R. KOTLOWSKI (By Invitation) *Hines, Ill.*
Introduced by Raymond J. Dern, M.D.

Twenty-four hour protein-bound radioactive iodine (PBI^{131}) determinations, done by a dialysis technique in 126 cases, by the trichloracetic acid (TCA) precipitation method in 151 cases, and by the anion-exchange resin column method in 128 cases, are compared to determine the relative reliability of the methods. All patients included had an established diagnosis of thyroid status obtained independently of PBI^{131} determination, so that the performance of the 3 methods employed could be tested against cases of known thyroid condition.

In the TCA group, 102 were euthyroid, 27 hyperthyroid, 22 hypothyroid; in the resin group, 99 were euthyroid, 12 hyperthyroid, 17 hypothyroid; and in the dialysis group, 99 were euthyroid, 10 hyperthyroid, and 17 hypothyroid.

Results for the TCA-euthyroid group had a mean PBI^{131} of 0.019 μc per liter plasma, with a S. D. of ± 0.012; hyperthyroid subjects had a mean of 0.151 μc per liter, with a S. D. of ± 0.129; and hypothyroid patients had a mean of 0.008 μc per liter, with a S. D. of ± 0.005.

The resin-euthyroid group had a mean PBI^{131} of 0.015 μc per liter with a S. D. of ± 0.010. Hyperthyroid subjects had a mean of 0.082 μc per liter, with a S. D. of ± 0.094, and hypothyroid persons had a mean of 0.007, with a S. D. of ± 0.003 μc per liter.

The dialysis-euthyroid group had a mean PBI^{131} of 0.013 μc per liter, with a S. D. of ± 0.005; the hyperthyroid subjects had a mean of 0.088 μc per liter, and a S. D. of ± 0.092;

while the hypothyroid group had a mean of 0.005 μc per liter, and a S. D. of ± 0.002 μc per liter.

The dialysis method is simply performed by placing 5 ml. of oxalated plasma obtained 24 hours after a 50 μc dose of I^{131} in a ¾ inch Visking bag and dialyzing in saline at 9° C. for 24 hours, rotating the bag at 60 r.p.m.

The data present evidence that the dialysis technique is more likely to differentiate euthyroid from hyperthyroid cases than the other 2 methods. A clinically useful degree of separation between euthyroid and hypothyroid cases is possible using the dialysis technique.

55. Identification of galactose-6-phosphate in galactosemic erythrocytes

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Galactosemia is a hereditary metabolic disorder characterized by vomiting, diarrhea, jaundice, poor weight gain, and malnutrition during early infancy. Mental retardation, cataracts, and cirrhosis of the liver may also be seen. In this condition, the absence of galactose-1-phosphate uridyl transferase results in the accumulation of galactose-1-phosphate (Gal-1-P) in the erythrocytes when galactose is contained in the diet. The present paper will present evidence on the presence of galactose-6-phosphate (Gal-6-P) during the utilization of galactose by galactosemic erythrocytes.

Erythrocytes of galactosemic patients were incubated with galactose and the phosphorylated sugars were isolated by an anion exchange resin column. The fraction containing the monophosphate esters were subjected to paper chromatography on a system of n-propanol-ammonia-water (6:3:1) before and after hydrolysis with 1N HCl at 100° C. for 10 minutes. Densities after staining with aniline hydrogen oxalate were observed which corresponded to authentic Gal-6-P in samples with and without hydrolysis, whereas Gal-1-P was absent after hydrolysis.

The material contained in the Gal-6-P fraction was isolated by preparative paper chromatography using the n-propanol-ammonia-water system and subjected to alkaline phosphatase and galactose oxidase. After alkaline phosphatase, chromatography of the hydrolysate revealed densities corresponding to galactose. The hydrolysate treated with galactose oxidase indicated the presence of galactose by the reduction of dianisidine in the presence of added peroxidase by hydrogen peroxide released by the oxidation of galactose.

Finally, galactosemic hemolysates were incubated with adenosine triphosphate, magnesium chloride, and 10 μmoles of galactose for 2 hours at 37° C., deproteinized, and chromatographed. Densities corresponding to Gal-6-P, Gal-1-P, and Gal-1,6-P were observed. Similar incubations carried out with 10 μmoles of Gal-6-P yielded Gal-1-P and Gal-1,6-P, but incubation with 10 μmoles Gal-1-P yielded neither Gal-6-P nor Gal-1,6-P. These data would suggest the participation of phosphoglucomutase during the utilization of galactose or a hexokinase phosphorylating galactose in the 6 position rather than a galactokinase phosphorylating in the 1 position.

56. Development and persistence of serum neutralization of a common cold virus

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(By Invitation), and GLORIA JOHNSON, B.S. (By Invitation)
Chicago, Ill.

From studies in volunteers we have shown data relating to the susceptibility and immunity of individuals to challenge with an infectious common cold agent as follows: (1) on the basis of incubation period and the clinical syndrome, it was possible to postulate that different

viruses were present in common cold secretions in different years, indicating that common colds are the result of an infection with one of several, perhaps many, different viruses; (2) neutralization of infectivity for volunteers by pooled human gamma globulin showed that these viruses had caused natural widespread infection in man, were antigenic, and elicited a neutralizing antibody response; and (3) individual immunity to specific virus infection was shown by the development of resistance to rechallenge with the same infectious secretion but not with another common cold virus.

To elucidate the appearance and persistence of serum neutralizing activity 2 volunteers were bled at 1, 6, 12, 18, and 24 months after an experimental challenge. The neutralizing activity of the serum for the challenge virus was tested in 275 other volunteers. The results showed insignificant protection from the serum collected at 1 month but good neutralizing activity at 6, 12, and 18 months after challenge. By 24 months the neutralizing capacity had weakened considerably. The retarded antibody response suggests that the common cold virus was either poorly antigenic or an entirely new antigenic experience for the host. The neutralizing activity of the serum remained high for at least 1 year after infection and could have afforded the specific immunity to reinfection observed in volunteers during this period. The decline in serum neutralizing capacity suggests a return of the host to a more susceptible state for reinfection and may mean that natural reinfection with this virus has not served as a booster stimulus. The results will be interpreted in the context of the frequency of the common viral respiratory infections.

57. Effect of moderately heavy exercise conducted in a hot, dry environment on the splanchnic hemodynamics of normal young ambulatory male volunteers

PAUL M. JACKSON, M.D. (By Invitation), ELMERICE TRAKS, M.D. (By Invitation), and SALVATORE M. SANCETTA, M.D. *Cleveland, Ohio*

Previous studies in this laboratory have demonstrated no significant change in splanchnic blood flow (EHBF/M^2) and a significant decrease in splanchnic vascular resistance of normal subjects and those with diseased left ventricles at rest following a 2 hour exposure to a relatively dry, warm (98° F.) ambient environment (humidity 40 per cent) (BSP extraction). Studies by others have demonstrated decreased EHBF and increased splanchnic vascular resistance in normal subjects after 10 minutes of moderately heavy exercise.

The effect of such exercise for 30 minutes (enough to triple the rate of resting total body oxygen uptake) at an external work effort of 2,550 feet, 1 pound per minute was investigated in 16 normal ambulatory volunteers ranging in age from 18 to 35 years. In 8 subjects control and exercise flows were determined 2 hours apart in a comfortable environment (73° F., 40 per cent humidity). In the other 8 subjects control data were obtained in a comfortable environment; the subjects were then exposed to the warm environment for 90 minutes, after which they exercised for 30 minutes, and blood samples for BSP analysis were drawn during the last 6 minutes of the exercise period.

Exercise in the comfortable environment produced significant increases in the heart rate and in BA-HVO_2 difference, and no significant changes in EHBF/M^2, splanchnic vascular resistance, $SMRO_2$, or arterial BP. The effects of similar exercise in the warm environment differed only in that a significant decrease was noted in EHBF/M^2 and a significant increase in splanchnic vascular resistance.

The data would indicate that the decreased EHBF and increased splanchnic vascular resistance noted by others during moderately heavy exercise may become compensated by secondary circulatory homeostatic readjustments when exercise is permitted to continue for at least 30 minutes. The added demands brought about by similar exercise in a warm environment may combine to prevent such a readjustment, thus resulting in a diversion of

blood to other vascular territories. It is also evident that studies designed to assess the effects of exercise on splanchnic blood flow in intact human beings must be interpreted in the light of the environment in which they are conducted as well as the duration of exercise.

58. Chronotropic effects of digitalis studied by direct perfusion of the sinus node

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(By Invitation) *Detroit, Mich.*

A study of the chronotropic effect of drugs is in essence a study of the behavior of the sinus node. Because this structure is richly innervated and subject to many extracardiac influences it is difficult in the intact heart to determine whether a drug has a direct or indirect effect on the cardiac pacemaker. An additional inotropic effect further obscures results. Digitalis exerts both inotropic and chronotropic effects, but it is uncertain how much direct effect on the sinus node is exerted by digitalis in the intact heart.

To examine this question a catheter was placed within the artery to the sinus node in 12 dogs, the hearts of which were exposed via a midline incision. This permitted perfusion of the sinus node and a small amount of adjacent atrial myocardium without perfusing the ventricular myocardium. With such localized perfusion it was possible to employ minute quantities of various drugs, observing their isolated effect on cardiac rate and rhythm. The sinus node could be arrested with 0.1 μg of acetylcholine (0.1 ml. volume) delivered within ½ second. Advantages of the preparation include preservation of the intact heart, including its own blood supply and innervation, and the direct delivery of pharmacologic agents through the sinus node's own artery. Postmortem studies included India ink and Hypaque injections with radiographs, to verify catheter position and determine the area perfused.

Various concentrations of acetyl-strophanthidin (0.1 to 1.0 μg per milliliter) and digoxin (0.5 to 25.0 μg per milliliter) were perfused through the sinus node for various lengths of time (10 to 180 seconds). At calculated "digitalizing" levels there was no significant effect on cardiac rate or rhythm. At "arrhythmogenic" and "lethal" levels there was increasing bradycardia and ultimate sinus arrest. These effects were initially irreversible, but after 1 to 2 hours of autoperfusion with blood from the femoral artery the sinus arrest could be transiently reversed with either epinephrine, atropine, potassium, or normal saline. Premedication with atropine did not prevent the effect of digitalis. Although "therapeutic" levels of digitalis had no significant chronotropic effect, these levels enhanced the negative chronotropic action of calcium, acetylcholine, and cold solutions.

59. Studies of individual patterns of urinary 17-ketosteroid fractions

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Employing a standardized technique for separating urinary 17-ketosteroids into 4 fractions (dehydroepiandrosterone, androsterone, etiocholanolone, and 11-oxygenated 17-ketosteroids), patterns of excretion of these steroids have been studied in 67 human subjects. In 22 subjects determinations have been made on 2 or more 24 hour urine collections made at intervals varying from successive days to 10 months under relatively basal conditions without treatment. In 30 subjects the effects of the continued administration of small doses of cortisone or related steroids upon the fractionation patterns have been studied, and in 4 subjects the effects of intravenous infusion of both small (2 units) and large (40 units) doses of ACTH upon these patterns have been determined.

Observations of special interest include: (1) each person appears to have his own characteristic pattern of 17-ketosteroid excretion under relatively basal conditions when not

receiving medication or experiencing unusual stress; (2) the administration of ACTH or cortisone does not necessarily result in a proportionate rise or fall of all 17-ketosteroid fractions, but different persons may have relatively large changes in some fractions with little or no change in others; (3) the continued administration of small doses of cortisone or related steroids does not result in an immediate decrease in excretion of steroid fractions to their ultimate new level, but decreases may be progressive for at least a month, the excretion of some fractions appearing to fall more rapidly than others.

Although the significance of the variations of individual patterns of 17-ketosteroid excretion is not known, it is possible that they may reflect differences in steroid metabolism that might influence response to stress or susceptibility to stress diseases. The differences in response of the fractions to stimulation or inhibition suggests that some pathways of steroid metabolism may be more responsive than others, and this, in turn, might affect response to stress in different persons.

60. Circulatory studies in the head

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Circulatory studies in the head are assuming increasing importance because of the following: (1) the effects of surgery on the skull and brain on the circulatory dynamics are not well understood; (2) there is an increasing recognition that the bones of the skull have measurable elasticity, particularly the roof of the orbit; (3) the roof of the orbit is so elastic that it causes a forward movement of the eye with each systole, thus it is probably concerned with the nutrition of the eye; (4) these studies indicate that the diastolic blood pressure of the brain as recorded through the eye may be different from that recorded in the arms or legs; (5) the contour of the arterial volume pulse recorded in the head is usually different from the contour of the arterial volume pulse recorded in the arms or legs; and (6) this method of study provides a method of comparison of the circulatory dynamics within the skull as compared with the circulatory dynamics in the arms or legs.

Recordings of the circulatory changes and changes in the size of the skull with cardiovascular activity have been made over the temporal area, the frontal area, and the intraorbital tissues in over 100 patients. These studies were made on normal adult patients, patients with cranial defects, intracranial lesions, various eye diseases, and various forms of cardiovascular disease. The studies were made as an office procedure by means of oscillography. Permanent optical recordings were made on 6 cm. electrocardiograph papers which were calibrated to 0.1 ml. in volume, and to 0.04 seconds in time.

The results show striking variations in adult normal patients, those with cranial defects, intracranial lesions, various eye lesions, and various cardiovascular diseases.

These studies show that the skull is elastic, and this elasticity varies from patient to patient. In view of these findings, the Monroe-Kelly doctrine will probably have to be modified to the extent that the accuracy of the doctrine will be related to the degree of elasticity of the skull.

61. A possible site of action of thyroid hormone

PHILIP C. JOHNSON, M.D., and TOMMIE REDDING, B.S.
(By Invitation) *Houston, Texas*

Mammalian erythrocytes, being devoid of mitochondria but containing both the hexose monophosphate shunt pathway and the Embdem-Meyerhof pathway of glucose oxidation, make convenient preparation for the study of the effects thyroid hormone has on these two metabolic pathways. Certain enzymes of the HMP shunt pathway have been shown to be increased in erythrocytes from thyroid-treated animals and in thyrotoxicosis.

Using C^{14} glucose in vitro, we have found that the HMP shunt pathway of glucose oxidation is depressed to 27 ± 6 per cent (mean ± S. E.) of normal in 10 thyrotoxic and 3 normal individuals treated for 2 days or more with triiodothyronine. As cure of the thyrotoxicosis occurs there is a return to normal values which roughly parallels clinical improve-

ment. A similar change was not found in the Embdem-Meyerhof pathway. Adrenelectomy augmented this depression but produced only a slight change in the HMP pathway (76 per cent of normal), in euthyroid animals. This depression was not influenced by the addition of glucose-6-phosphatase dehydrogenase, TPN, or ATP to the incubation flask.

We conclude that the increase in the enzymes used in the hexose monophosphate shunt pathway of glucose oxidation and found in thyrotoxicosis, e.g., glucose-6-phosphate dehydrogenase, is compensatory to a depression in activity of this pathway produced by thyroid hormone through a now unknown mechanism which would seem not to be a decrease in TPN. Erythrocytes, unlike other tissues, show no increase in the Embdem-Meyerhof pathway during thyrotoxicosis. This would mean that the metabolic effect of thyroid hormone cannot be ascribed only to changes in mitochondrial permeability and that this hormone would seem to have sites of action which differ in various tissues.

62. A new variant of hemoglobin M disease: Hemoglobin $M_{Chicago}$

A. M. JOSEPHSON, M.D., H. G. WIENSTEIN, M.S. (By Invitation), V. J. YAKULIS, B.S. (By Invitation), L. SINGER, M.S. (By Invitation), and P. HELLER, M.D. *Chicago, Ill.*

Congenital methemoglobinemia may be produced by either an enzymatic defect or a structural abnormality of the globin moiety of the hemoglobin molecule. The former variety of this genetic disorder is transmitted as an autosomal recessive gene and the enzyme concerned is diaphorase. In the latter type of disturbance the characteristic mode of inheritance is as an autosomal dominant. Five previously reported varieties of hemoglobin M are to be found in the literature. This report deals with a sixth deviant labeled hemoglobin $M_{Chicago}$. The group of hemoglobins M demonstrate heterogeneity in certain of their physicochemical criteria. These differences concern the spectroscopic pattern and their reactivity with ligands. The propositus, a 15-month-old girl, presented with cyanosis. There was no evidence of a congenital cardiac abnormality or pulmonary disease. Routine physical examination was noncontributory with the exception of the cyanosis. The peripheral blood count indicated a moderately severe reticulocytosis. Determination of diaphorase activity was normal. When the hemolyzate was oxidized with potassium ferricyanide and subjected to electrophoresis at pH 7.1 (phosphate buffer), a grayish-green fraction, not present in the normal control, was demonstrable. Elution of this fraction revealed spectroscopic bands at 494 and 598 mμ and a distinct minimum at 563 mμ. Studies on the mother, father, and the one sibling, aged 8 years, were entirely normal. Additional studies indicate that the use of tris-EDTA-boric acid buffer at pH 8.9 on agar gel allowed for separation of the M fraction in an untreated hemolyzate. Agar gel electrophoresis at pH 7.1 of an oxidized hemolyzate gave separation equally as good as that seen with the starch block technique. Cr^{51} survival time of the patient's own erythrocytes indicated a moderately severe hemolytic process with a $T\frac{1}{2}$ of 19 days. Presentation of the spectroscopic and electrophoretic findings and discussion of the genetics of this particular disorder will be made.

63. The effects of ethylalcohol administration on urinary excretion of magnesium and other electrolytes in alcoholic and normal subjects

JOHN M. KALBFLEISCH, M.D. (By Invitation), ROBERT D. LINDEMAN, M.D. (By Invitation), and WILLIAM O. SMITH, M.D. *Oklahoma City, Okla.*

Hypomagnesemia is frequently seen in chronic alcoholism, but little is known regarding the responsible mechanisms. Five alcoholic subjects, 2 with documented hypomagnesemia, and 2 normal persons were studied. All subjects were on a normal hospital diet, and fasted over-

night. Water loading was achieved with 20 ml. of water per kilogram of body weight and maintained by replacing fluid volume excreted. When an adequate water diuresis was obtained, serum and urinary sodium, potassium, calcium, magnesium, chloride, phosphate, osmolality, inulin, and PAH were measured during three successive 20 minute control periods. Subsequently, 30 ml. of 100 per cent ethyl alcohol was given orally with a maintenance intravenous infusion of 5 per cent ethyl alcohol. Following a 20 minute equilibration period, measurements during 3 successive 20 minute test periods were compared with the control periods.

All subjects showed from 90 to 357 per cent (mean 180 per cent) increase in magnesium excretion and from 46 to 173 per cent (mean 98 per cent) increase in calcium excretion which was independent of the water diuresis. Urinary sodium and chloride showed no consistent changes. Total solute excretion was slightly increased, potassium excretion was markedly decreased, and phosphate excretion was slightly decreased. Serum values were not significantly altered from the control values except for a consistent increase in osmolality (mean increase of 14.3 ± 5.1 mOsm. per liter).

These findings indicate that ethanol induces a marked increase in excretion of magnesium. The effect is more pronounced in magnesium-deficient subjects, suggesting that increased urinary excretion of magnesium may be accentuated by magnesium deficiency. Acetazolamide administered intravenously to one subject did not block the magnesium diuresis induced by ethanol. This increased urinary excretion of magnesium is independent of water diuresis, PAH administration, and the carbonic anhydrase transport system.

In view of reports by previous investigators that: (1) there is an increase in production and urinary excretion of lactate following ethanol ingestion, and (2) there is an increase in urinary magnesium following lactate infusion, it seems likely that the magnesium diuresis observed in our studies may be related to increased lactate production.

64. A continuous in vitro method of monitoring dynamic phenomena in biologic systems

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JOHN J. IMARISIO, M.D. *Hines, Ill.*

Introduced by Irving A. Friedman, M.D.

Kinetic studies of protein binding, enzymatic lysis, and similar biologic dynamics indicate a need for more precision and sensitivity. A radiosotope technique has been devised for this purpose, to evaluate relative transference rates, binding capacities, accumulation gradients, and dissociation properties of gamma-labeled permeable ions and molecules as related to nonpermeable molecules, cells, tissues. or organisms.

To evaluate the various phenomena above, a small Visking dialysis bag, containing the test substance in 1 ml. volume, is immersed in a test tube of solution. The solution in the tube is continually replaced from a reservoir at a constant rate adequate for quantitative removal of gamma-labeled particles diffusing from the bag. In the system described, a rate of flow in excess of 5 ml. per minute will prevent significant back diffusion into the Visking bag.

The test tube and dialysis cell are monitored in a scintillation well detector, in a temperature-controlled chest. The output of the detector system is recorded by a scaler-digital printer system.

A simple application of this device containing a solution of a permeable radioactive particle within the dialysis bag allows determination of unidirectional transference rate. This rate may be observed as influenced by physical variables, e.g., temperature, pH, membrane properties, ionic charge, concentration gradient, etc.

Nonpermeable substance placed within the bag, such as protein molecules, blood cells, or tissue fragments, may alter dialysis rate by interaction between the static component and the radioactive permeable component. Retention of the radioactivity in the bag or concentration and retention against a gradient from the flow system allows study of binding

capacity and accumulation gradient. The decrease of escape rate from the system is proportional to the dissociation of complexing between the permeable and nonpermeable components. Interaction of a nonradioactive third component may activate or depress a lytic enzyme or metabolically active substance, with resultant variation in dialysis rate.

This continuous monitored dialysis-perfusion device has been developed to facilitate the quantitation of binding and dissociation characteristics of various biochemical and biologic systems in vitro with minimal interference by the observation technique.

65. The measurement of pain: A new approach to an old problem

ERIC C. KAST, M.D. *Chicago, Ill.*
Introduced by Hyman J. Zimmerman, M.D.

The shortcomings of existing methods for the measurement of pain are discussed briefly.

In order to make the assessment of analgesics in particular, and the measurement of pain in general, more objective and precise, the following concepts were developed: (1) A pathologic pain can be compared by the subject with a simultaneously occurring experimental pain of increasing intensity, and the point at which the two pains are equal can be precisely determined by the subject. When this end point occurs, we may assume that the pathologic pain of unknown intensity is equal to the measured experimental pain. Thus we have an indirect but objective way to quantitate pain. For this purpose a device was constructed, producing a measured mechanical pain. This device is operated by the patient himself. He activates the pain production and, upon reaching the end point, terminates it himself. He is, however, not aware of the pain intensity he is indicating. A graphic writing device records the intensity of the mechanical pain production. (2) The end point, when the pathologic pain and the experimental pain are perceived as equal, makes itself known to the subject in a variety of ways. The two pains may be suddenly perceived as one pain sensation, topographically indistinguishable. We called this phenomenon "pain fusion." In other cases the subject may feel his attention shift suddenly from the pathologic to the experimental pain. We measured many pains with this method, including labor pains, phantom limb pains, pelvic pains due to pelvic inflammatory disease, postoperative wound pain, and herpes zoster pain. We also followed the pain of a postoperative course through its crescendo and lessening. The effect of various analgesic drugs was tested, including meperidine HCl, acetylsalicylic acid, codeine phosphate, and an APC compound. The results are reported and a theory of pain and the mechanism of analgesic action implied by this study is presented.

66. A comparative study of hospital and community gram-negative rods

DAVID M. KESSNER, M.D. (By Invitation), and
MARK H. LEPPER, M.D. *Chicago, Ill.*

During the past decade evidence has been accumulated to document an increasing incidence and severity of gram-negative infections in the hospitalized population. The aim of this study is to examine the hypothesis that there exists a population of gram-negative rods within the hospital which differ from community strains by virtue of their increased resistance to antibiotic drugs.

The hospital population under consideration was characterized regarding age, sex, and race distribution, socioeconomic level, and the incidence and sites of infection. In the study group 143 infections were deemed present and 54 per cent were due to gram-negative rods. Subsequent to this survey, all gram-negative organisms isolated from hospitalized patients were obtained and the charts of these patients reviewed in order to judge the presence or

absence of infection and the type: hospital acquired, community, or indeterminate. Gram-negative organisms were also cultured from a group of individuals attending a cancer detection clinic not affiliated with a hospital.

Gram-negative bacteria were obtained from 1,900 hospitalized patients over a 9 month period. Two-fifths of the cultures were of the urinary tract, while soft tissue, sputum, and nasopharynx each accounted for 10 per cent. Four genera, *Escherichia coli*, Proteus, Pseudomonas, and Klebsiella-Aerobacter comprised 30, 22, 19, and 18 per cent, respectively, of the isolated strains, while cultures from 250 "community" patients revealed a higher proportion of *E. coli*. The organisms from the individuals adjudged to have a "hospital" or "community" acquired infection or carrier state were identified as to group, family, and species, employing the methods and nomenclature of Edwards and Ewing. Tube dilution and disc sensitivity tests were performed for each of 5 antibiotics. No significant difference between 57 community and 13 hospital *E. coli* strains was observed with any antibiotic; however, in the case of Klebsiella, the percentage of hospital strains resistant to streptomycin, chloramphenicol, and tetracycline was significantly higher than that for the community strains.

These data lend credence to the concept that a hospital population of drug-resistant Klebsiella do exist and suggest that with the use of more sensitive biologic markers a similar phenomenon may be discovered for other gram-negative genera.

67. Demonstration that hormonally determined serum inhibitors of insulin action on skeletal muscle are ineffective on adipose tissue

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In the course of studies delineating the factors regulating insulin responsiveness in skeletal muscle and adipose tissue, the importance of circulating insulin inhibitors was assessed. The present report presents evidence indicating the existence of hormonally determined serum factors which inhibit the action of insulin on skeletal muscle but not on adipose tissue.

Numerous investigators, using the isolated rat diaphragm, have demonstrated that the sera of animals rendered alloxan-diabetic or given growth hormone and/or hydrocortisone possess marked insulin-inhibiting activity. In the present study, serum insulin inhibitory activity was assessed with the rat epididymal fat pad assay technique by determining the per cent recovery of a known quantity of insulin added to serum. A test dose of 250 microunits of crystalline beef insulin was selected because it produced a readily detectable yet submaximal effect when added to human and rat sera and the response elicited was in that portion of the dose-response curve affording maximal analytic precision.

The serum of rats given growth hormone (0.1 mg. per 100 grams body weight daily × 5 days) or made diabetic by either hydrocortisone (5 mg. per 100 grams body weight daily × 5 days) or alloxan monohydrate failed to demonstrate any insulin inhibitory activity on the epididymal fat pad. These studies were then extended to human subjects. No demonstrable insulin inhibitory activity in the fat pad system was observed in the sera of 10 normal adults, 12 people in whom the onset of diabetes occurred during adulthood (none having ever received insulin), 2 patients with active acromegaly and diabetes, and 2 patients with hyperadrenocorticism and diabetes.

The failure to detect inhibitory activity on adipose tissue in the serum of rats in which fat pads demonstrate depressed sensitivity to insulin suggests that tissue responsiveness to insulin is determined by metabolic changes at the cellular level. The existence of serum factors which inhibit the action of insulin on skeletal muscle but not on adipose tissue may be of significance in the pathogenesis of diabetes mellitus beginning in adulthood and explain in part some of the pathophysiologic changes, e.g., obesity, observed in this disease.

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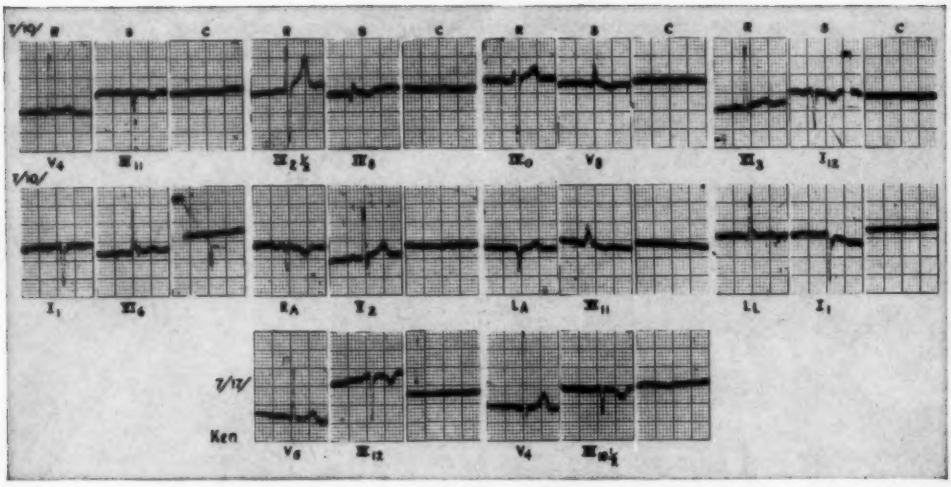

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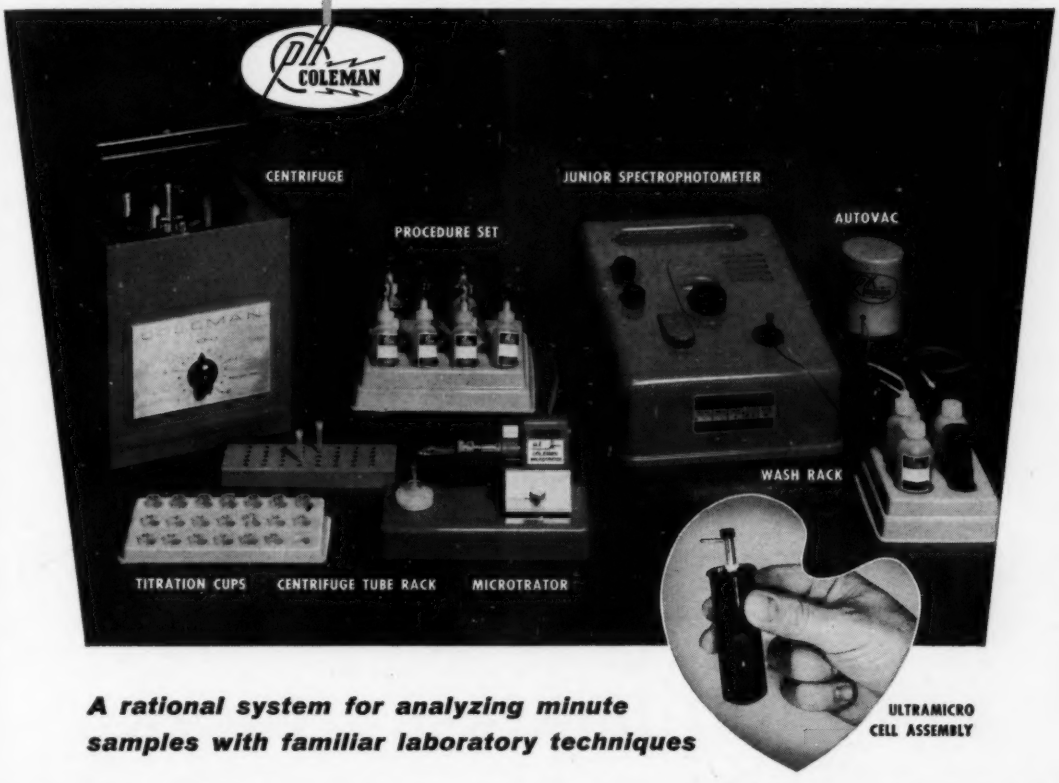

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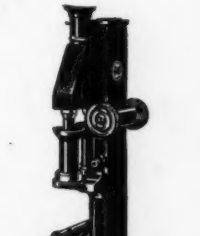

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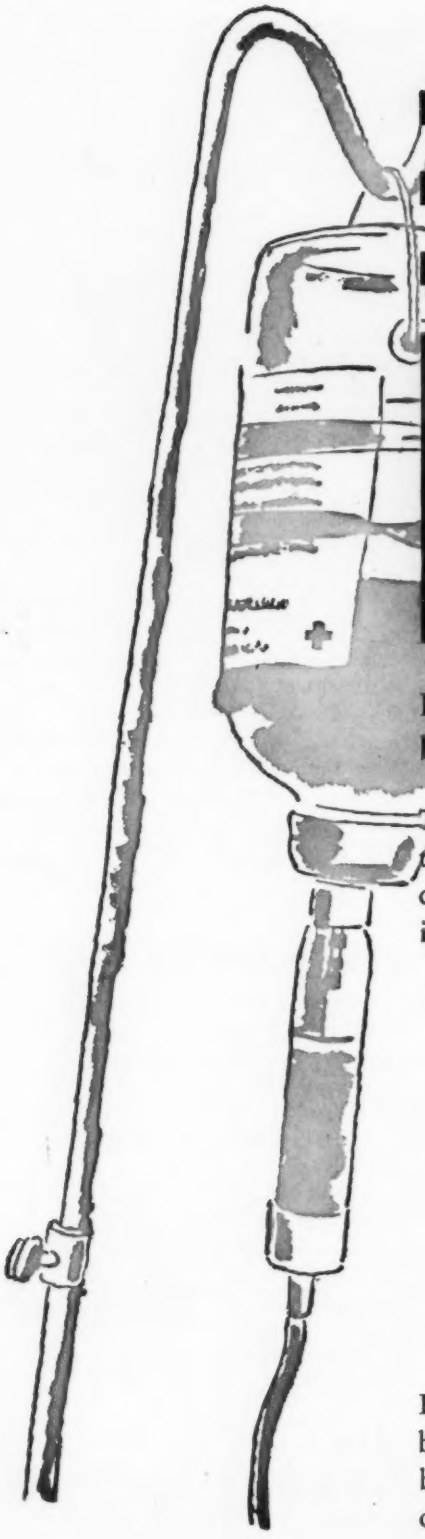

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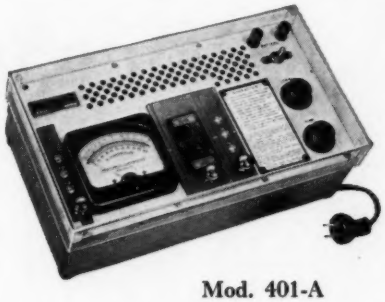

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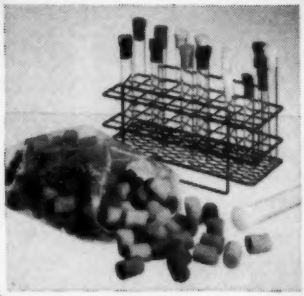

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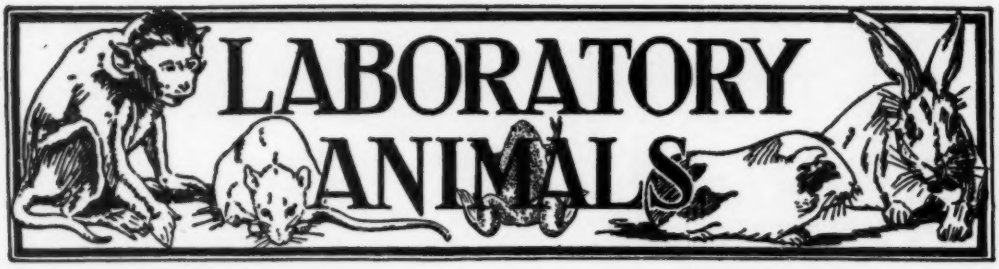

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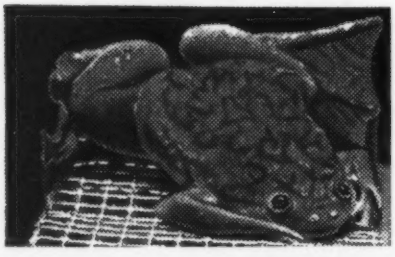

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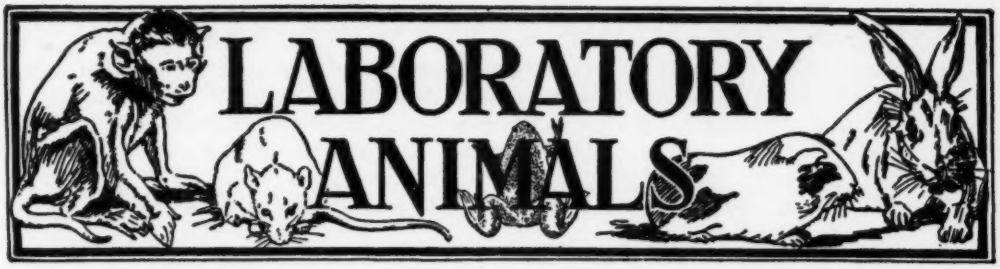

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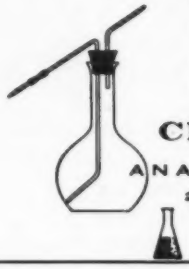

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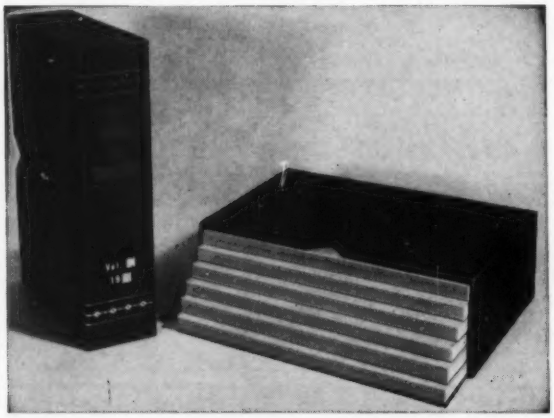

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While every precaution is taken to insure accuracy, we cannot guarantee against the possibility of an occasional change or omission in the preparation of this index.

add confidence

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ANTI-HUMAN SERUM

FOR THE COOMBS ANTI-GLOBULIN TEST

Ortho Pharmaceutical Corporation

RARITAN, NEW JERSEY

Ortho

the AutoAnalyzer Announcer

being intelligence on current developments in the automation of wet chemistry

two pumps for the price of one (almost)

17 Our laboratory informs us of successful field tests... ganging-up 12 pumping tubes on *one* proportioning pump. The happy results? ...Quadruple and multiple analyses for electrolytes, glucose/cholesterol, etc. use only one pump in place of the two as reported in the last issue. What's more incredible... our laboratory has added up to 15 tubes on one platen, without loss of AutoAnalyzer efficiency.

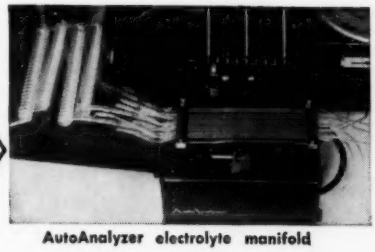
new flow-cell design offers more analyses per hour... by drawing off the air bubbles immediately before the sample enters the new AutoAnalyzer flow cell, it may now feed directly into the viewing window. Shortened dwelltime and less mixing in the flow cell increases washout so you may run B-U-Ns, for example, at a brisk one-a-minute rate, instead of the previous 40 per hour. *New* speed sacrifices nothing of *old* accuracy... you get the same results, augmented by about half again as many in the same time. Further... now fluid is *pumped* out of the cell so that the large gravity feed drain tube (and possibility of stoppage) are eliminated from the colorimeter.

bursting at the seams... technicon expanding again... a new Technicon building program (second we've been forced to in as many years) will increase our manufacturing and service facilities by a third. Means we'll be better able to keep abreast of the swelling tide of demand for AutoAnalyzers... maybe even stockpile against your orders. At the same time, raising our sights on pure research, we're hard-pressed for talented technical personnel. Any reader interested in a challenging job (or knowing anyone else who may be) please get in touch with us right now. *Write, Wire, Phone.*

this fall... "join the club" on automation in your own back yard... AutoAnalyzer users will welcome our new program of technical meetings in certain selected cities this fall... *workshops* where they can brush up on newly automated analytical techniques, learn better ways, fuller means. If you're already an AutoAnalyzer user, you'll be advised by mail. If not, you're still welcome: We ask only that you clear with us in advance. Write for the coming schedule.

Na + K on urine as well as serum . . . we've now adapted the flame attachment for the AutoAnalyzer so a range from zero to 200 milliequivalents of Na or K can be read as full scale measurements. Practically no limit now to the range of biological materials measurable for Na and K.

1881 alumni to date... and no end in sight... something new under the sun is Technicon *college*, started barely three years ago to teach AutoAnalyzer users how to utilize and get the most out of their machines, it has blossomed into a full-fledged school boasting an array of equipment that many a formal academy would envy... among other things, thirty complete AutoAnalyzers to serve classes numbering as many students. They come from all over the world... join together in happy harmony for a week's intensive training... go forth armed with a new appreciation of the expanding potential of automated chemistry, and new competence for dealing with it... *Makes us proud.*



AutoAnalyzer electrolyte manifold

more and more interesting papers... at the recent meeting of the American Society of Clinical Chemists, several papers of interest to AutoAnalyzer users and members, were presented. A review on current trends and new tools in the automation of clinical chemistry procedures was presented by Andre Ferrari, Director of Research. Another, a review of the philosophy of the automation and what it means to the clinical chemistry laboratory was presented by Mr. E. C. Whitehead of our company.

our face is red . . . for many years we have mistakenly been receiving Dialyzer membrane material from the manufacturer under the assumption that it was not coated. Imagine our chagrin when recently, the manufacturer quite casually informed us that the membranes he was supplying were coated. An uncoated membrane not only allows the same amount of protein to be held back in dialysis, but as a bonus, allows more of the defusable material to come across. This gives much better sensitivity and better wash between samples. This new uncoated membrane material will now be supplied routinely. *Free samples* have been sent to all users... *have you received yours?*

branded sample cups foil foulups . . . simplify bookkeeping . . . from now on, every AutoAnalyzer sample cup will wear a lifetime number permanently embossed into it, *clear, unmistakable, ineradicable*. Serial numbers, running 0 to 100 will be easy to match up to serial record of specimen receipt in the laboratory.

true glucose controversy laid to rest? Ardent researchers seeking pinpoint precision will hail a new AutoAnalyzer procedure for glucose-oxidase determinations. Brilliant results (we fondly hope) should settle once and for all the *true* glucose debate. Tests can run at 40 per hour. *Send for a paper on this new technique.*

TECHNICON INSTRUMENTS CORPORATION
Research Park, Chauncey, N. Y.

Gentlemen:
Please send me the TECHNICON AUTOANALYZER BROCHURE plus data on the items checked.

☐ 17 ☐ 18 ☐ 19 ☐ 20 ☐ 21 ☐ 22 ☐ 23 ☐ 24 ☐ 25 ☐ 26

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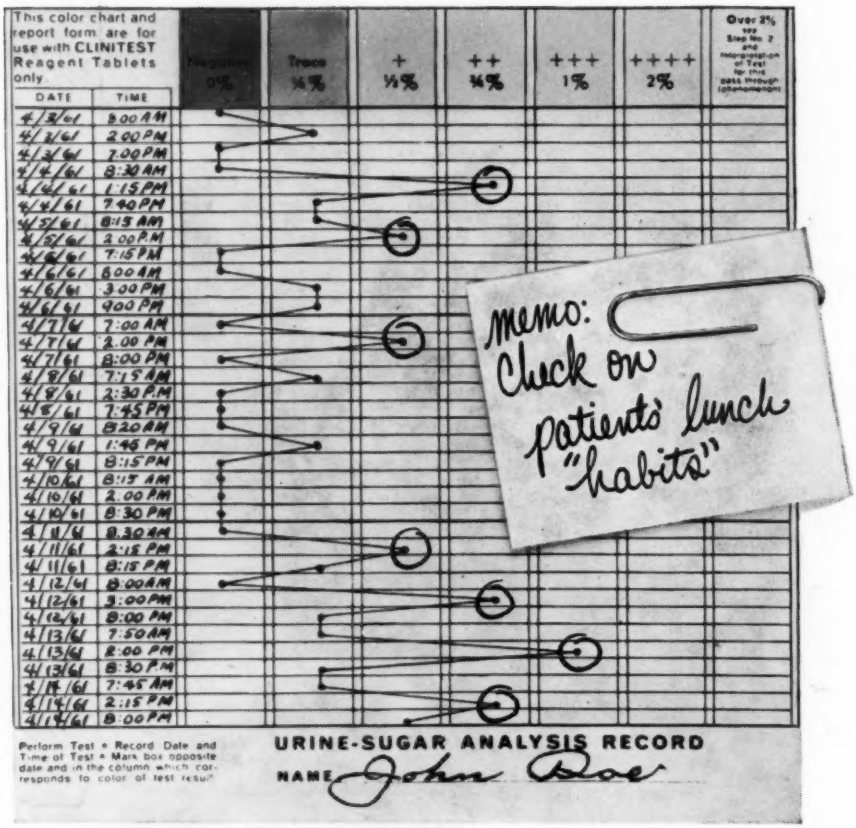
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